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Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) Optimization and Validation for Ricin Protein

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Haluan kiittää koko VERIFIN:in henkilökuntaa saamastani avusta ja tuesta tätä työtä tehdessäni. Erityiskiitos professori Paula Vanniselle tästä upeasta mahdollisuudesta ja työni ohjaamisesta. Lämpimästi kiitän myös Anne Puustista ja Marja-Leena Rapinojaa saamastani avusta. Kiitos kuuluu myös yliopettaja Jukka Niiraselle työni ohjauksesta ja saamastani tuesta.

Andrew, thank you for your support, love and understanding.

Tekijä Otsikko Sivumäärä Aika	Nina Donnell Kerrosmaisen Entsyymivälitteisen Immunosorbenttimäärityksen (ELISA) optimointi ja validointi risiiniproteiinille 35 sivua + 8 liitettä 1.12.2017
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Risiini kuuluu Kemiallisen aseen kieltosopimuksen (CWC) listan 1 kemikaaleihin. Sille ei ole tunnettua vastalääkettä ja se on myös terrorismiriski myrkyllisyytensä vuoksi. Mahdollisen väärinkäytön uhan vuoksi on tärkeää, että risiinille on tarkka havainnointimenetelmä. Tässä opinnäytetyössä optimoitiin ja validoitiin entsymaattinen risiinin määritysmenetelmä, kerrosmainen entsyymivälitteinen immunosorbenttimääritys (kerros ELISA) Kemiallisen aseen kieltosopimuksen instituutille (VERIFIN).

Kerros ELISA -menetelmän optimointi keskittyi vasta-aineiden biotinylointiin, puskuriliuoksiin, vasta-ainekonsentraatioihin ja entsyymikonjugaatin konsentraatioon. Vasta-aine- ja entsyymikonjugaatin konsentraatiot määritettiin titraamalla ja parhaat konsentraatiot valittiin signaali-kohinasuhteen perusteella. Validointi keskittyi nollarajan (LOB), toteamisrajan (LOD), sisäisen analyysin ja analyysien väliseen tarkkuuteen, saantoon ja spesifisyyteen. LOB:n, LOD:n ja analyysin tarkkuuden tulokset määritettiin useasta ELISA-tuloksesta. Saantokokeissa testattiin ELISA-menetelmää kolmella eri näytematriisilla: maidolla, punaviinillä ja vehnäjauhoilla. Menetelmän spesifisyys testattiin käyttämällä kahta risiinin kaltaista proteiinia, agglutiniinia ja abriinia.

Menetelmän optimointi onnistui ja menetelmällä pystyttiin havaitsemaan vaivattomasti risiinikonsentraatiot 0,03 % BSA ja 0,1 % TritonX PBS liuoksessa 7,6 pg/ml asti. Vasta-aineiden optimoidut konsentraatiot olivat päällystykseen 2,5 µg/ml ja havainnointiin 0,8 µg/ml. Entsyymikonjugaatille optimoitiin 1 ng/ml pitoisuus. LOB oli 2,4 pg/ml ja LOD oli 2,9 pg/ml. Sisäisen analyysin tarkkuus vaihteli 3,9 % ja 105 % välillä. Analyysien välinen tarkkuus vaihteli 14 % ja 35 % välillä. Näytematriisit haittasivat risiini ELISAa, ja näiden osalta saannot jäivät alhaisiksi. Risiini ELISA ristireagoi agglutiniinin kanssa, kun agglutiniinia oli näytteessä 25 pg/ml. Abriini ei sitoutunut käytettyihin risiinivasta-aineisiin.

Kerros ELISA risiinille toimi hyvin, kun käytössä oli optimoitu näyte matriisi, 0,03 % BSA ja 0,1 % TritonX PBS:sä, mutta käytettäessä kolmea testattua matriisia oli niillä huomattava vaikutus analyysiin ja risiinin havainnointiin. Validointitulokset osoittavat, että menetelmässä on vielä useita osa-alueita joita tulisi kehittää.

Avainsanat	Risiini, kerrosmainen entsyymivälitteinen immunosorbenttimääritys, kerros ELISA, biotiini, biotinylaatio, validointi, immunoanalyysin optimointi, vasta-aine, LOB, LOD, saanto, ristireaktio

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Ricin is listed in the Chemical Weapons Convention (CWC) as a schedule 1 toxic chemical with no known antidote and threat of terrorism due to its toxic nature. Because of the potential misuse, a method for precise detection of ricin is crucial. In this study enzymatic ricin detection method, Sandwich Enzyme-Linked Immunosorbent Assay (sandwich ELISA), was optimized and validated for the Finnish Institute for Verification of the Chemical Weapons Convention (VERIFIN).

Sandwich ELISA method optimization is focused on antibody biotinylation, multiple buffer solutions, antibody concentrations and conjugate enzyme concentration. Antibody and conjugate enzyme concentrations were determined using titration and concentrations where ideal signal-to-noise ratio were chosen. Validation focused on limit of blank (LOB), limit of detection (LOD), intra- and inter-assay precision, recovery and specificity. LOB, LOD and assay precisions were determined from multiple ELISA results. Recovery of the ELISA method was tested using three matrices: milk, red wine and wheat flour. Specificity was tested using two ricin like proteins agglutinin and abrin.

Method optimization was successful detecting ricin in 0.03 % BSA 0.1 % TritonX in PBS solution comfortably down to 7.6 pg/ml. Optimized concentrations for antibodies were for coating 2.5 µg/ml, detection 0.8 µg/ml and conjugate enzyme was optimized to 1 ng/ml. LOB was 2.4 pg/ml and LOD was 2.9 pg/ml. Intra-assay precision was lowest at 3.9 % and highest at 105 %, inter-assay precision low was 14 % and a high of 35 %. Sample matrices disturbed the ricin ELISA determination and recovery from three matrices were low. No cross reactivity for abrin was found. However, agglutinin from 25 ng/ml onwards was detected.

Sandwich ELISA for ricin works well when using optimized sample matrix, 0.03 % BSA 0.1 % TritonX in PBS, but the other three matrices had substantial effect on the assay and ricin detection. Validation results suggest that the assay has still a lot to be improve upon.

Keywords	Ricin, sandwich enzyme-linked immunosorbent assay, sandwich ELISA, biotin, biotinylation, validation, immunoassay optimization, antibody, LOB, LOD, recovery, cross reactivity

Contents

Abbreviations

BBC	British Broadcasting Corporation
BSA	Bovine Serum Albumin
BWC	Biological Weapons Convention
CGE	Capillary Gel Electrophoresis
CV	Coefficient of Variation
CW	Chemical Weapon
CWA	Chemical Warfare Agent
CWC	Chemical Weapons Convention
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
EuroBioTox	European programme for the establishment of validated procedures for the detection and identification of biological toxins
HRP	Horseradish Peroxidase
in vivo	Testing on living organisms or cells in their environment
in vitro	Testing in test tube
kDa	Kilo Dalton
KOH	Potassium Hydroxide
LC-ESI/MS	Liquid Chromatography–Electrospray Ionization Mass Spectrometry
LFA	Lateral–Flow Assays

LOB	Limit of Blank
LOD	Limit of Detection
mAb	Monoclonal Antibody
MALDI-TOF/MS	Matrix–Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry
NHS	N-hydroxysuccinimide
OPCW	Organisation for Prohibition of Chemical Weapons
pAb	Polyclonal Antibody
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline and Tween 20
RIP	Ribosome Inactivating Protein
RKI	Robert Koch Institute
RT	Room Temperature
RTA	Ricin toxin A
RTB	Ricin toxin B
SA-HRP	Streptavidin–Horseradish Peroxidase
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TMB	3,3', 5,5;-tetramethylbenzidine

UN	United Nations
VERIFIN	Finnish Institute for Verification of the Chemical Weapons Convention
4PL	Four Parameter Logistic

1 Introduction

This study was conducted at the Finnish Institute for Verification of the Chemical Weapons Convention (VERIFIN), which is an institute at the Department of Chemistry at the University of Helsinki. VERIFIN was founded in 1994 as a continuance for the 1973 Chemical Weapons research project (CW). Fundamental missions of VERIFIN are to develop analytical methods for Chemical Warfare Agents (CWA), aiding Organisation for Prohibition of Chemical Weapons (OPCW) and United Nations (UN) and training chemists from developing nations. VERIFIN also functions as the National Authority of Finland for the Chemical Weapons Convention (CWC), performs tasks specified by the Ministry for the Foreign Affairs of Finland, conducts research and teaches post-graduates. Working as the designated laboratory for OPCW, VERIFIN can receive authentic environmental and biomedical samples. [1]

Objective for this study was to optimize enzymatic method of ricin detection for the institute. The study concentrated on method optimization and validation for sandwich ELISA (Enzyme-linked immunosorbent assay). Since there are no commercial kits available for ricin ELISA detection the purpose was to develop a method to obtain reliable results in ricin determination. Purpose was also to provide VERIFIN with detailed working instructions for the use of sandwich ELISA. This study was lightly associated within EuroBioTox project, a European project focusing on assessment and improvement of toxin detection methods. EuroBioTox is aiming for a network of European laboratories with universal way of handling biotoxin incidents. [2]

Ricinus communis plant is universally used source for castor oil, a raw material in several products such as cosmetics, lubricants and pharmaceuticals. Ricin is very accessible and easily obtained. Its high toxicity makes it a prospective agent for bioterrorism. Previously ricin has been used for bioterrorism in the case of Georgi Markov and threat letters containing ricin sent to the U.S. Senate, White House and President Obama. Ricin is listed as a prohibited substance in the CWC and the Biological Weapons Convention (BWC). Because of potential misuse of ricin, a method for rapid and precise detection of ricin is essential. [3]

2 Chemical Weapons Convention

The goal set out by the Convention is to eliminate all weapons of mass destruction. The Convention prohibits all member states, so called state parties (currently 192), of development, production, stockpiling, transferring or using chemical weapons. States parties are obliged to execute the prohibition within their jurisdiction. Within the CWC all state parties have agreed to destroy all chemical weapons they may possess and weapons left abandoned in their territory. State Parties are obliged to create a verification administrative system for some toxic chemicals and their precursors to ensure that those chemicals are used in ways that are not prohibited. [4]

Ricin belongs to the Schedule 1 toxic chemicals of CWC. Schedule 1 chemicals have been used, developed, produced or stockpiled as a chemical weapon. A chemical can be listed under the Schedule 1 when it possesses lethal toxicity or is incapacitating, has a structure similar to other listed chemicals, can be used as a precursor for listed chemicals and has little or no use for purposes not prohibited under the Convention. [4]

Even ricin was found not effective enough for military use, as it has been used for multiple attacks and incidents. There have been 12 known incidents involving ricin, including Minnesota militia members intention to kill government officials in 1994 and Kurdish Sunni Islamists testing ricin on animals for use in terrorist acts in the United Kingdom [5, 158; 6, 282]. Presumably the most well-known ricin incident is the murder of Bulgarian writer Georgi Markov. He had defected to the West and was working for the British Broadcasting Corporation (BBC) World Service in London. September 7th in 1978 he was waiting for bus near Waterloo Bridge when he was stabbed in the calf with an umbrella containing a platinum pellet loaded with ricin. [6, 283; 7, 141] Markov developed a high fever and was submitted to hospital where he died on the 11th of September 1978 [8]. Cause of death was a complete block of the cardiac conductive tissue [6, 283]. Postmortem revealed pulmonary edema, hemorrhages on heart, intestines, and lymph nodes [8]. Two weeks prior Bulgarian exile Vladimir Kostov was attacked in Paris metro using an almost identical method to the Markov case, but the pellet did not penetrate deeply and Kostov survived [6, 283].

Because of these incidents in the past, it is possible that ricin will be used again and therefore research of ricin detection and determination is essential.

3 Biological Weapons

Biological weapons, as BWC Article 1 states, refer to the means of spreading any biological agents and toxins. These means are munitions, bombs, aircraft spray tanks and other devices. [5, 3; 9]

Biological weapons have been used throughout the history of warfare. Polluting wells using animal carcasses and disposal of plague victim corpses inside enemy city walls (siege of Caffa in 1346). More recent cases of biological weapons use include the smallpox infected blankets that British army gave to the American Indian tribe in 1763. [5, 3–5]

The first agreement of prohibition of chemical and biological weapons was made on 1675 in Strasbourg. France and Germany agreed not to use poison bullets. [5] After 200 years a prohibition of “poisons and poisoned weapons” was agreed at Brussels Conference in 1874. In 1899 European delegates agreed on not using projectiles for spreading of “asphyxiating or deleterious gases”. Even though these prior agreements were signed in 1907, it did not prevent use of chemical weapons by both sides in World War I, nor use of biological agents. [5, 3–5]

In modern war biological weapons have been verifiably used in two cases: Japan in Manchuria from 1934 to 1945 and Germany in World War I. In Manchuria the Japanese army developed a biological program and intentionally spread epidemics by contaminating food and water and using plague-infected fleas. During World War I Germans were trying to infect pack animals with anthrax and glanders. [5, 4–5]

From World War I and onwards multiple countries including France, the United Kingdom, the United States of America and Soviet Union have been developing different biological weapons [6]. Early on scientists realized that bombs with bacteria were close to impossible to produce, because of the heat when detonating. Use of virulent agents and toxins, especially botulinum and ricin, were researched further. Botulinum was difficult to produce in large quantities and ricin was found to be not effective enough for military use. [5, 14–15]

After the Cold War, a plan for BWC came to life. BWC wanted similar protocols and methods as CWC had achieved. USA, Russia and China expressed their resistance in

the beginning for multiple reasons. In the end an agreement was found. [5, 148] Today BWC has 175 state-parties [10].

4 Ribosome Inactivating Protein (RIP)

4.1 Ricin

Ricin, highly toxic plant toxin, originates from seeds of castor bean plant, *Ricinus communis*, the source of castor oil (Figure 1) [11; 12]. Castor oil is used in multiple applications, such as lubricants, cosmetics and detergents [11]. Origination of the plant is most likely from Africa and now it grows wild and as an ornament in tropical and subtropical regions [13]. Weight of the castor bean consist of approximately 1–5% of ricin protein [14].

Figure 1. *Ricinus communis* plant in bloom (a) and seeds of *R. communis* cultivars (b) from left to right: *R. c. zanzibariensis*, *R. c. zanzibariensis*, *R. c. green giant*, *R. c. zanzibariensis*, *R. c. carmencita*, *R. c. india*, *R. c. tanzania*. [13]

The water-soluble protein was originally identified in 1888 by Stillmark [3; 6, 1; 13] and it consist of two polypeptide chains, making it an AB toxin. Chains A and B are linked together with disulfide bond (Figure 2). Ricin belongs to a large gene family that also encodes seven full-length ricin or ricin like proteins and several shorter genes with unknown properties. These seven full-length proteins have similar protein synthesis inhibition properties as ricin. [3] Ricin is type 2 ribosome-inactivating proteins (RIP-II) in size approximately 64 kDa. [12] B-chain has lectin activity and binds to different oligosaccharide residues located on the cell surface. After entering the cell, ricin protein uses the Golgi network for travel in endoplasmic reticulum where the two chains are reduced. After reduction into two chains the catalytically active A-chain, acting as RNA

N-glycosidase, gets transported to cytosol where it binds to ribosomal stalk of the ribosome and removes one adenine from GAGA tetraloop of 28S rRNA [3; 12; 13]. Removal of the adenine prevents binding of elongation factor 2 inhibiting protein biosynthesis and leads to cell death.

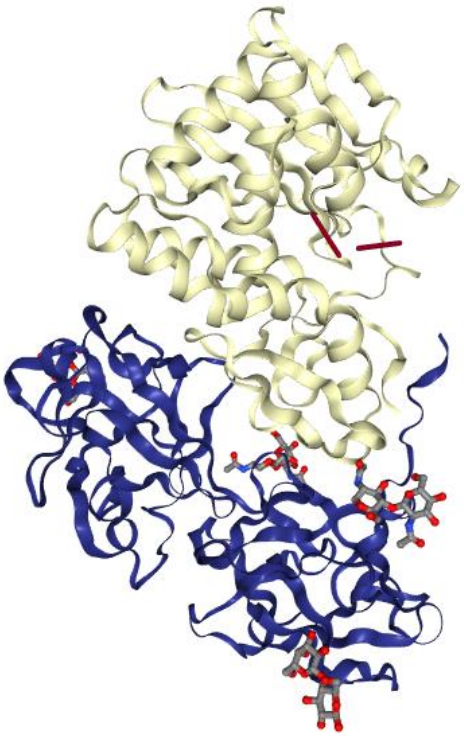

Figure 2. Crystal structure of ricin bound with dinucleotide ApG (PDB ID: 3RTJ). Two chains shown with different colours, A chain as white and B chain as blue. [15]

Ricin toxicity when ingested is estimated to be 1–20 mg/kg body weight and inhaled or injected 1–10 µg/kg body weight. [3; 12] After ingestion symptoms of ricin intoxication arise from 4 to 6 hours but it may take as long as 10 hours. The first symptoms are universal and can consist of abdominal pain, nausea and vomiting. These symptoms are usually followed by cramps, diarrhea, fever, sore throat, dehydration, dilation of pupils, headache, low blood pressure, heartburn, internal bleeding of digestive system and failure of the kidneys, liver and spleen. Death can follow after three or more days when the vascular system fails. There are no known antidotes or vaccines available for ricin intoxication and treatment for the intoxication is based on supporting the body´s natural response. [16]

Due to previous military and bioterrorism use ricin is a prohibited substance under the CWC and the BWC. OPCW rigorously regulates and controls possession or purification of the protein. Despite its destructive uses the catalytic A-chain of ricin has been one of the first examples of a toxin conjoined to monoclonal antibodies (mAb) against cell surface proteins. It has been also tested experimentally for the treatment of various cancers. [17]

Characterization is hindered by another protein present in seeds called *R. communis* agglutinin, RCA120. Another problem with detection is two existing ricin isoforms D and E (Figure 3). [3]

Figure 3. Amino acid sequence homologies between ricin isoforms D and E, agglutinin and abrin [18].

4.2 Agglutinin, RCA120

Agglutinin, also known as RCA120 is a heterotetrameric 120 kDa protein consisting of two heterodimers similar to that of ricin connected via disulfide bond. Ricin and agglutinin share a very similar sequence, 93 % in A chain and 84% in B chain (Figure 3). Nevertheless, ricin is 300-fold more toxic than RCA120. [3]

4.3 Abrin

Abrin is a toxic protein in the seeds of *Abrus precatorius*, also known as rosary peas, jequirity bean, and crab's eye. Molecular weight of abrin is approximately 65 kDa and it has two subunits A and B similar to ricin. Abrin is RIP-II protein and has high amino acid sequence homology with ricin (Figure 3). Inhaled toxicity for humans is estimated at 0.1–1 µg/kg. First symptoms are vomiting and diarrhea following bloody diarrhea and black stools. [19]

4.4 Methods for Identification and Detection of Ricin

There are several methods for ricin identification and detection, such as immunological, spectrometric, functional and molecular approaches, but no universal approach has been agreed on. [3]

Methods for determination of protein composition, size and purity are sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and capillary gel electrophoresis (CGE) [3]. Both methods are based on the molecular size [20].

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) and liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS) are both used for tryptic fingerprinting and sequencing. LC-ESI/MS is also utilized for molecular weight determination and MALDI-TOF/MS for analysing trace contaminants. [3] Both methods use mass spectrometry for identification, but the ionization technique differentiates. Electrical energy is exploited in ESI, where ions in the liquid are dispersed as minuscule charged droplets into gaseous phase [21]. MALDI-

TOF applies a laser that heats the sample matrix, which is vaporized and various charged ions are released [22].

Enzyme-linked immunosorbent assay (ELISA) is used for detection of ricin. Because in this method ricin and antibodies with other reagents create a colour reaction, it can be used with spectrometric methods to measure ricin concentration. For faster screening lateral-flow assays (LFA) are used and can measure ricin up to 1-50 ng/ml [3; 13]. Ricin antibody reaction can also be applied to Western Blot. [3] In Western blot separation of proteins is based on molecular weight. Separated proteins are transferred to a membrane and membrane incubated with specific antibodies. [23]

Cytotoxicity assay measures functional activity. It is based on cell viability and death when ricin is introduced to the cell culture plate. Functional activity measurements indicate presence of intact ricin molecule. Ricin molecule needs to be intact with its both chains to be toxic. [3]

5 Enzyme-Linked Immunosorbent Assay, ELISA

Enzyme-linked immunosorbent assays are plate based assays used for protein, antibody, peptide and hormone quantification and detection. It is assay with multiple perks as it needs lesser volumes of reagents, adapts to different detection systems and needed reagents and equipment are accessible for most laboratories. In ELISA, antigen can bind to the assay plate or be captured by antibody immobilized on the plate. [24] Immobilization and binding of reagents make it easy to plan and perform and non-specifically bound material can be easily washed away, making it an excellent assay for specific analyte detection and quantification. [25] In ELISA method, antibodies detect the antigen directly with labelled primary antibody or indirectly with labelled secondary antibody. [24] Measurable detection, usually a colour, is achieved with conjugate and substrate in incubation and measured using different methods. The most essential component of the assay is a specific interaction between the antibody and antigen. [25]

Common ELISA formats are direct and indirect. Both capture and detection can be direct or indirect and capture and detection formats can be mixed together (Figure 4). When antigen is immobilized to microtiter plate, it is called a direct capture. For indirect capture microtiter plate is first coated with specific antibody and antigen that binds to the

antibody. In direct detection primary antibody with conjugated reporter binds to antigen and substrate that reacts with conjugated reporter is added for detection. When primary antibody attaches to antigen which then will be recognised by secondary antibody with attached conjugate reporter, is called indirect detection. In indirect detection substrate is added, but when compared to direct detection there are more conjugate reporters for one antibody and the signal is stronger. [25]

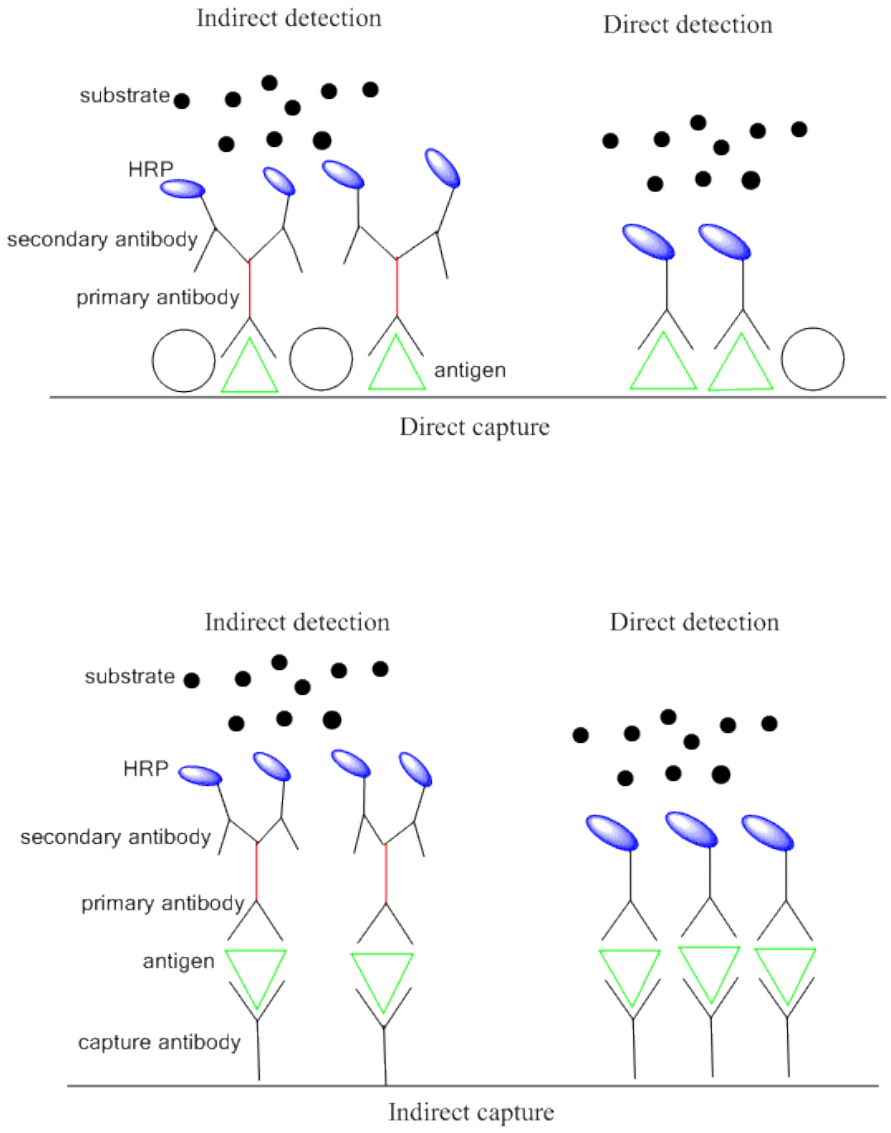

Figure 4. Mixture of direct and indirect capture and detection using horseradish peroxidase (HRP).

5.1 Sandwich ELISA

In sandwich ELISA, antigen is bound between two antibodies, the capture antibody and the detection antibody [24]. Antibodies used for the assay can be either monoclonal or polyclonal. Monoclonal recognize single epitope, thus making quantification easier and it is usually used as a detection antibody. Polyclonal binds considerable amounts of antigen and is often used as a capture antibody. Antigens used for sandwich ELISA must contain at least two sites able to bind antibodies. [26] Sandwich method is more sensitive because of indirect capture and indirect detection. In sandwich ELISA the capture antibody is immobilized to microtiter plate. Antigen binds to the capture antibody and labelled detection antibody binds to antigen. Conjugate binds to label used in detection antibody and reacts with substrate forming measurable product (Figure 5). [25] Multiple sandwich ELISA formats have been developed for ricin detection [24]. Sandwich ELISA can be challenging to optimize [26].

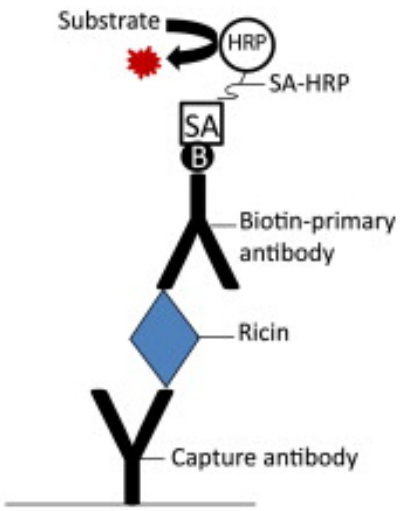

Figure 5. Sandwich ELISA with biotinylated detection antibody and streptavidin-horse radish peroxidase (SA-HRP) conjugate [24]

5.2 Antibodies

When infectious pathogens or toxins are found, mammal B cells secrete and produce antibodies. Antibodies bind to the antigen and with other immune system proteins they inactivate foreign antigens. B cells produce specific antibodies to match antigens epitopes. Single antigen can contain multiple different epitopes, but B cell can only

produce one kind of antibody matching only one epitope. Because of this B cells differentiate to produce different antibodies against different epitopes. [27, 119]

There are different types of antibodies, polyclonal (pAb), monoclonal and recombinant antibodies. These antibodies differentiate from each other in production. Polyclonal antibodies are produced by multiple different B cells against same antigen. Polyclonal antibodies bind to different epitopes on the antigen. Monoclonal antibodies are produced using B cells cloned from one B cell. All produced antibodies are then similar and bind to only one epitope of certain antigen. Monoclonal antibodies are more specific than polyclonal. Recombinant antibodies are combination of mouse mAbs with human segments. These are used clinically for multiple purposes. [27, 119–120]

6 Biotin

Biotin is also known as vitamin B7, vitamin H and coenzyme R. It is nontoxic and water-soluble, important coenzyme involved in carbon dioxide transfer in carboxylase reactions. [28; 29; 30] Numerous plant and animal tissues such as liver, brains, yolks and corn kernels are rich with biotin [30]. Because of biotins small size of 244 Da it does not usually alter proteins biological activities [29]. Valeric acid chain of biotin molecule (Figure 6) works as a spot for adding different reactive groups that promote biotin labelling to other molecules [30]. Biotin binds with high affinity to streptavidin and avidin making it an attractive component for molecular tests and immunoassays [28; 29].

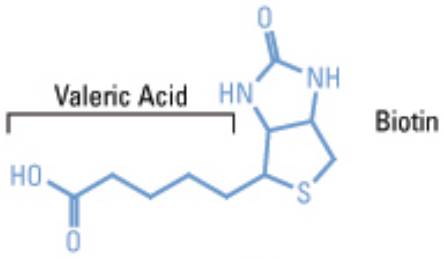

Figure 6. Biotin molecule structure [31].

6.1 Biotinylation

The process attaching biotin with chemical or enzymatic methods to antibodies, proteins or other macromolecules is called biotinylation [29; 31]. Chemical methods are more superior compared to enzymatic methods and can be performed *in vivo* and *in vitro*. For this reason, when biotinylation is mentioned, it refers to the chemical methods. [31]

Reagents available for biotinylation have all similar features. Reagents can be targeted for certain functional groups as sulfhydryls, primary amines, carbohydrates and carboxyls. Targeting biotin to right functional group prevents target molecule inactivation. Moieties are attached to valeric acid chain with a structure called spacer arm. The length of the spacer arm increases (long spacer arm) or decreases (short spacer arm) depending on availability of biotin for avidin binding. [31]

Besides spacer arm length there are other factors affecting biotinylation. Solubility of the biotinylation reagent affects ability to label targets. Reagent solubility is hinged on reactive moiety (part of functional group in molecule), spacer arm or a combination. Biotinylation reagents cleavability/reversibility affects the ability to purify labelled proteins by cleaving biotin or reversing biotin-avidin complex. Lastly, and perhaps the most substantial factor, is targeting the functional groups. To reduce potential interference of biotinylation reagent conjugating to amino acids that regulate normal protein function, there are many accessible moieties. Selection of right moiety is crucial for successful biotinylation. In this study biotin was targeted for primary amines (Figure 7), the most commonly targeted functional group. N-hydroxysuccinimide (NHS) forms bonds with primary amines and do not carry a charge. Therefore NHS-esters are usually altered to be water-soluble by sulfonating N-hydroxysuccinimide ring to form sulfo-NHS. [31]

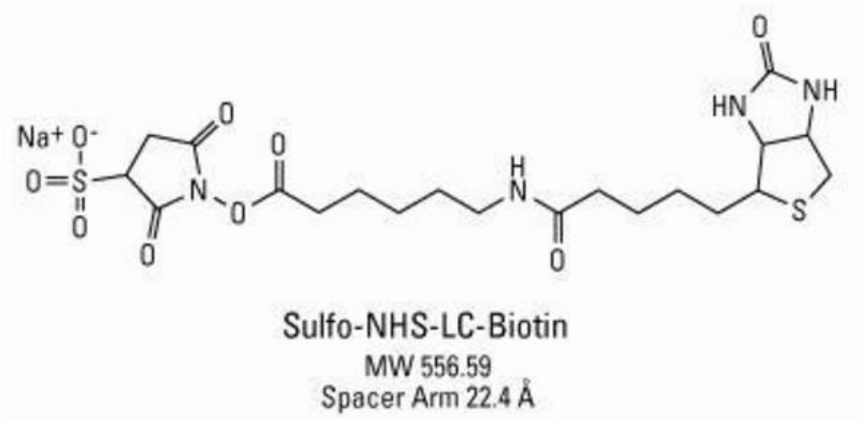

Figure 7. Biotin used on the thesis analysis [29].

6.2 Biotin Quantitation

Knowing the amount of biotin bound to a sample can be useful and aid optimization of assay system. Common method for measuring labelled biotin amounts is 4'-hydroxyazobenzene-2-carboxylic acid dye also known as HABA. In this method HABA is covalently bound to avidin when there is no biotin around. When biotin is available it replaces HABA. This is because its association constant for biotin-avidin is higher than HABA-avidin. Both mixes display absorbance at wavelength 500 nm, but higher absorbance is available when HABA is bound to avidin than when biotin replaces it. When measured absorbances are deducted the amount of biotin bound in the sample can be calculated. New methods for quantitation use the same principle as above, but use more sensitive fluorescent reporters. [31]

7 Immunoassay Optimization

Key to precise ELISA assay is in three steps: method development, optimization and validation. Method development starts with deciding of antibodies, antigens, enzymes and method of detection to use. In optimization, all these elements get tested and titrated to achieve ideal assay performance. Part of optimization is also making the most effective compromises between convenience and analytical capability. Lastly, validation measures developed and optimized method. [32, 381]

ELISA assay optimization will need multiple experiments to achieve the best results. The primary target for ELISA is to identify antigen, have a most favorable environment for binding affinities and assay kinetics and grant consistent results with high sensitivity and specificity. To meet these requirements, many factors must be balanced. [32, 390]

Every different ELISA method has its unique reagent formulation. Commercial reagents can shorten development time, but may also lead to possible problems because the reagents are not optimized. Thus, every assay reagent must be determined empirically for optimal concentrations. Popular reagent concentration determination is checkerboard titration, also known as a two-dimensional serial dilution. It is often used for achieving optimized dilutions. In this titration of two components concentration is varied, making it a pattern resembling its name. [32, 391]

The signal-to-noise ratio is the ratio of signal of sample compared to level of noise. Noise is the signal when no analyte is in the sample. When the signal-to-noise ratio enhances, the assay will be more effective detecting small quantities of antigen. To enhance the signal-to-noise ratio you can either reduce noise or increase signal. [32, 391]

7.1 Buffers

Buffers are the foundation of ELISA. Factors to be considered in buffer formulation are buffer system, pH, ionic strength, salt, detergent, blocking agents, proteins, preservatives, and other additives. Detergents in buffers can reduce nonspecific binding, but all detergents do not go well with all antigens. Use of a detergent, high salt and certain pH range may be inhibited by antigens conformational epitope. Buffers can be used to stabilize antigen–antibody complex and can result in better surface complementarity. This is important to the accessibility mobility and antigenicity of an antigen. Antigens flexibility allows its epitope to assume more easily suitable configuration in the antibody-binding site. [32, 391]

7.1.1 Blocking Buffer

Blocking unoccupied sites is essential for preventing nonspecific binding. Antibody antigen pairs can require different blocking buffers because of their different characteristics. Used blocker depends on the antigen and on the enzyme conjugate. Well

selected and tested blocking buffer can increase specificity and sensitivity. [32, 388] Best sign for right blocking buffer choice is signal-to-noise ratio. Excessive blocking can obscure antibody-antigen interactions resulting in lower signal-to-noise ratio. [32, 391] For blocking buffer optimization, different buffer solutions and concentrations are prepared to experiment with [33].

7.2 Capture Antibody and Detection Antibody Concentration

Optimum antibody concentration is critical. It improves assay performance and may save funds. Optimizing the concentrations is the key part of the assays sensitivity, right amounts will yield best results detecting very low concentrations of antigen. [32, 387] In optimization multiple different dilutions of capture and detection antibody are made and tested for the best result. Ideal results are with strong signal and low background [33].

7.3 Standard Diluent and Sample Volume

Standard diluent should be as close as possible to sample matrix if not the same matrix. Range for standard curve and linearity of dilution should be dynamic. If these are not met diluent should be a changed. [33]

Sample volume should be defined by minimal interference and matrix effect. Reaction of antibody–antigen develops at the solid–liquid interface. This makes it difficult to determine exact reaction volume. Larger volume can increase assay sensitivity, but it might lower linearity. [32, 391]

7.4 Enzyme Conjugate Concentration

Most commonly used enzyme conjugate is horse radish peroxidase (HRP). It produces coloured, luminescent and fluorometric derivative when incubated with a substrate. It generates strong signal for detection in short incubation time and works in broad pH range. Stability of the enzyme can be affected by nonionic detergents. [32, 389] For best results optimizing enzyme conjugate concentration is done as before, multiple dilutions are tested for optimum results [33].

7.5 Signal Detection

It is important to choose the right substrate to match enzyme conjugate. The choice of substrate to use depends on sensitivity of the substrate solution and instrument used for detecting. For optimization several substrates can be experimented with. [32, 389; 33] The other parts for optimization are addition of the stopping reagent and correct wavelengths to match and measure the substrate catalysis [32, 390].

7.6 Kinetics and Incubation

After optimizing assay reagents some experiments with incubation may still make the assay better. Aim is to achieve a high signal-to-noise ratio not just maximize the signal. Assay precision and sensitivity are important. Incubation times can be made shorter with higher concentrations of conjugate, but this can cause the prize of the assay to increase and result in weak signal generation. With long incubation time nonspecific signal generation is possible. Sandwich formats usually require shorter incubation times because of the use of higher capture antibody and conjugate concentrations. [32, 390]

Optimization of the incubation time usually begins with determination how time and temperature have an effect on nonspecific binding and signal intensity. Nonspecific binding can be reduced by altering incubation time and use of more specific monoclonal antibodies (mAbs) produce a higher signal-to-noise ratio. Assay cross reactivity decreases with incubation time and increasing the incubation temperature. [32, 390]

Balance is essential in kinetics. Factors such as reaction volume, conjugate mass, number of steps and matrix effects should be optimized same time with kinetics. Kinetics are reliant on pH, ionic strength, and temperature, particularly ion content and pH can have affect assay kinetics. [32, 390]

8 Validation

It is a process of establishing an analytical requirement and confirming that the method under examination has capacities consistent with application requirements [34, 8].

8.1 Limit of Blank and Limit of Detection

Limit of blank (LOB) is the highest analyte concentration predicted to be found when sample contains no analyte. Blank sample without analyte can produce a signal that can be similar to a signal of sample containing low concentrations of analyte. [35]

Limit of detection (LOD) is the lowest concentration that can be detected from the background by the method at a specified level of confidence. LOD is also known as sensitivity, minimum detectable value, detection limit and CCβ. [34, 20; 36] LOD should be calculated using preferably blank samples, samples containing very low concentration of analyte or using LOB. [34, 21; 35].

8.2 Intra-Assay and Inter-Assay Precision

Assay replication is important for attaining reliable assessment of method performance components like precision. Validation associated with replicate analysis should be designed to consider variations predicted during routine use. [34, 35]

Intra-assay precision demonstrates the replicability of wells in single assay plate. This will establish that samples give comparable results. [36]

Inter-assay precision should be below 10 % and it shows replicability of assays done during multiple different days. This will show that the assay will be consistent and produce comparable results over time. [36]

8.3 Recovery

In recovery different sample matrices are examined for possible disturbance in analyte detection. Matrices can have interfering factors that can change measured analyte results. Recovery validation is done by spiking different matrices with known amounts of analyte. If average recovery is between 80 % and 120 % it is considered that analysed matrix has nominal effect on the assay and quantification of the analyte. [36]

8.4 Specificity

This is defined as the extent to which the method can be utilized to determine analyte of interest without interferences from other analytes of similar behaviour. It is also known as cross reactivity and selectivity. [34, 8; 36] Cross reactivity can lead to inaccurate results such as false positives [36]. In this study specificity is seen as a cross reactivity and was determined using proteins similar to ricin. Proteins were abrin and RCA120 agglutinin.

9 Methods and Materials

9.1 Work Safety

Working with highly toxic material requires attention in work safety. It is crucial to work using proper protective equipment and devices and make sure that toxic material does not contaminate or pose a threat to worker or other personnel. All work with ricin must be done in laminar or biosafety cabinet. Protective gloves and coat must be worn at all times and gloves changed every time you remove your hands from laminar. Everything that comes into contact with ricin must be decontaminated using solution consisting two parts 10 % potassium hydroxide (KOH) and one-part ethanol.

9.2 Chemicals and Materials

Reagents used in this optimization and validation are found in Table 1.

Table 1. Used reagents.

	Reagents	**Supplier**	**Product Code**
Sandwich ELISA	R109 monoclonal antibody, B chain	Robert Koch Institute	
	R18 monoclonal antibody, A chain	Robert Koch Institute	
	Phosphate buffered saline, PBS	BioRad	1610780
	Bovine Serum Albumin Heat Shock Reagent Grade Powder, BSA	VWR	422351S
	Sodium carbonate, Na_2CO_3	Sigma Aldrich	S7795-500G
	Sodium bicarbonate, $NaHCO_3$	Sigma Aldrich	S8875-500G
	Triton X-100	BioRad	161-0407
	Tween 20	Merck	8.22184.0500
	Pierce Streptavidin Poly-HRP	Thermo Scientific	21140
	1-StepTM Ultra TMB-ELISA	Thermo Scientific	34028
	Sulfuric acid, H_2SO_4	Merck	016-020-00-8
Biotinylation	R18 monoclonal antibody, A-chain	Robert Koch Institute	
	PBS	BioRad	1610780
	EZ-Link Sulfo-NHS-LC-Biotin	Thermo Scientific	21327
	Pierce Biotin Quantitation Kit	Thermo Scientific	28005
	HABA/Avidin premix		
	Biotinylated HRP		
Lowry assay	Albumin Standard	Thermo Scientific	23209
	DC Protein Assay Reagent B	BioRad	500-0114
	DC Protein Assay Reagent A	BioRad	500-0113
Antigens	Purified ricin	Robert Koch Institute	
	Purified RCA120	Robert Koch Institute	
	Abrin	VERIFIN	

Reagents used during the method optimizing are found in Table 2. These are the reagents that did not work with the method or were inferior compared to chosen reagents.

Table 2. Discarded reagents.

	Reagents	**Supplier**	**Product Code**
biotinylation	EZ-Link Sulfo-NHS-Biotin	Thermo Scientific	21326
	Biotin (type A) Fast Conjugation Kit	Abcam	ab201795
	Biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccimide ester	Sigma Aldrich	B3295
	(+)-Biotin N-hydroxysuccinimide ester	Sigma Aldrich	H1759-5MG
	Ethanolamine	Sigma Aldrich	398136-25ML
	Dimethyl sulfoxide	Sigma Aldrich	D2650-5x5ML
blocking	BlockerTM Casein	Thermo Scientific	37528
antibodies	RA999 monoclonal antibody, A chain	HyTest	2R1
	RB999 monoclonal antibody, B chain	HyTest	2R1

Apparatuses used in this study are found in Table 3.

Table 3. Used devices.

Devices and materials	
Thermo Scientific Multiskan Go	UV/Vis spectrophotometer
Eppendorf ThermoMixer C	Shaker
Thermo Scientific Heraus Fresco 21	Centrifuge
Thermo Scientific Finnpipette F1	Multichannel pipette
Fisherbrand Elite 1-10 µl, 10-100 µl, 100-1000 µl	Pipettes
Amico Ultra MCWO	Centrifugal filters
Nunc MaxiSorp	96 microtiter plate
Waters, Adhesive Plate Seal	Plate seals

9.3 Antibody Biotinylation and Quantitation

Monoclonal antibody R18 for ricin A chain was biotin labelled for the sandwich ELISA. Biotinylation was completed using EZ-Link Sulfo-NHS-LC-Biotin from Thermo Scientific [29]. Biotin concentrations were quantitated using Thermo Scientifics Pierce Biotin Quantitation Kit [37].

Antibody was diluted to concentration 100 µg/500µl (200 µg/ml) using phosphate buffered saline (PBS). Biotin was prepared just before the use by adding 180 µl pure water to the vial. Volume of biotin needed for antibody biotinylation was calculated using the equation provided in the instructions [29]. After pipetting the needed volume solution was incubated in room temperature (RT) for 30 minutes.

Before quantitation excess biotin was removed using Amico Ultra centrifugal filters. Filters were washed before use. Biotinylated antibody solution was added to the vial and centrifuged for 10 minutes at 14 000 g. Solution was rinsed with 500 µl of PBS twice and centrifuged as before. After removal of biotin PBS was added up to desired volume and filter was turned upside down and centrifuged for 2 minutes 1000 g.

HABA/Avid mix was prepared adding 100 µl of pure water and mixed thoroughly. 160 µl of PBS was added on 96-well microtiter plate and 20 µl of HABA/Avid mix on top of it. Absorbance was measured using wavelength 500 nm. Blanks, controls and samples were added in volumes of 20 µl and absorbance measured as before. Results were calculated using given equations from the instructions [37].

9.4 Antibody Dilution

Two monoclonal antibodies were used, R109 against ricin toxin B (RTB) and R18 against ricin toxin A (RTA). The Robert Koch Institute kindly provided both antibodies. Capture antibody R109 was diluted with carbonate bicarbonate buffer pH 9.6 to concentration 2.5 µg/ml. Detection antibody was diluted with 1 % bovine serum albumin in phosphate buffered saline (1% BSA in PBS) to concentration 0.8 µg/ml. Antibody concentrations were optimized.

9.5 Ricin Standard Preparation

Standards were diluted from ricin standard received graciously from Robert Koch Institute. Dilutions were done with PBS 0.03% BSA 0.1% TritonX. Prepared standard concentrations after optimization were 7550 ng/ml, 755 ng/ml, 75.5 ng/ml, 7.55 ng/ml, 0.755 ng/ml, 0.0755 ng/ml and 0.00755 ng/ml.

9.6 Sandwich ELISA Protocol

100 µl of diluted capture antibody R109, mAb for ricin B chain, was added to Nunc MaxiSorp microtiter plate. The plate was covered with adhesive plate seal and incubated with shaking +4 °C overnight. Without removing capture antibody 200 µl of 1% BSA in PBS was added and plate was incubated with shaking at +37 °C for two hours. Solution

was removed by gently tapping plate on tissue paper and washed 3 times with 300 µl PBS 0.05% Tween (PBST). Ricin standards were diluted with 0.03% BSA 0.1% TritonX in PBS, 100 µl of solution was added and incubated with shaking two hours in +37 °C. Ricin standards were removed as before taking into account solution toxicity and decontamination. Plate was washed 3 times with 300 µl PBST. Biotinylated detection antibody R18, mAb for ricin A chain, was pipetted in volume 100 µl and incubated with shaking in +37 °C for two hours. Washing performed as previous. 100 µl of secondary antibody conjugate poly-HRP, was pipetted and plate was incubated for one hour with shaking at +37 °C. Washing was performed as before and 100 µl of chromogenic substrate 3,3', 5,5;-tetramethylbenzidine (TMB) was added. Plate was incubated with shaking in dark for 30 minutes at RT. Reaction was stopped using 50 µl 2 M H_2SO_4 and absorbance was read in wavelength 450 nm within 30 minutes of stopping the reaction.

9.7 Validation

Validation was done using results from optimized sandwich ELISA method. Standards, blanks and samples were all done in triplicates or duplicates during multiple days. Standards and blanks were diluted using 0.03 % BSA 0.1 % TritonX in PBS.

9.7.1 Recovery

Three different matrices were used in this analysis. Matrices were milk, red wine and flour. Matrices were spiked with ricin up to concentrations 0 ng/ml, 0.00755 ng/ml, 0.0755 ng/ml, 0.755 ng/ml, 7.55 ng/ml, 75.5 ng/ml, 755 ng/ml and 7550 ng/ml.

Milk and wine were tested with two dilution solutions, the matrix itself and in PBS 0.03 % BSA 0.1 % TritonX. Wine was also tested after pH change. Wines pH was changed to 8.08 using NaOH. pH changed wine was then diluted using same matrix to concentrations 0 ng/ml, 0.0755 ng/ml, 0.755 ng/ml, 7.55 ng/ml, 755 ng/ml and 7550 ng/ml.

With flour matrix an extraction was made with two solutions, 2 % acetic acid and PBST. 0.1 g flour was weighed and spiked with ricin. Then 1 ml of extraction solution was added and incubated in RT for 30 minutes. Solution was removed and diluted with matrix as mentioned before.

9.7.2 Specificity

Two other protein toxins RCA120 and abrin were analysed using optimized sandwich ELISA for ricin. Assay was done as before but tested concentrations were for RCA120 1000 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, 10 ng/ml, 5 ng/ml, 1 ng/ml and 0 ng/ml. For abrin concentrations were approximately 4000 ng/ml, 400 ng/ml, 40 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml, 1 ng/ml and 0 ng/ml.

10 Results

10.1 Antibody Biotinylation and Quantitation

EZ-Link Sulfo-NHS-Biotin was the first biotin used in the biotinylation of antibodies. It had been used successfully in earlier sandwich ELISA, but did not work in this study. Antibodies biotinylated with sulfo-NHS-biotin yielded lower absorption values, which could have been due to the short spacer arm hindering the attaching of biotin to antibodies.

The second biotin used was Biotin (type A) Fast Conjugation Kit. It had only reagents for three biotinylations and in comparison to other kits it was expensive. Results were similar to other discarded biotins.

The third biotin used was (+)-Biotin N-hydroxysuccinimide ester. In this biotinylation the biotin was diluted to dimethyl sulfoxide (DMSO) and ethanolamine was used to bind excess biotin after biotinylation. Antibodies bind with this biotin did not give higher OD450 values than 0.14.

The fourth biotin used was Biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccimide ester. Biotin was diluted in DMSO. Biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccimide ester had longest spacer arm out of the five biotins. OD450 results using this biotin can be found in Appendix 1. Absorption was similar to EZ-Link Sulfo-NHS-LC-Biotin.

Biotin chosen for use in this study was EZ-Link Sulfo-NHS-LC-Biotin. It has relatively long spacer arm and easy to use no weigh -format. Biotin is attached to the antibodies

easily, giving proper biotin molecule quantities during quantitation. Antibodies biotinylated with EZ-Link Sulfo-NHS-LC-Biotin gave high absorption results and continued working through rest of the optimization.

At the beginning of this study the assay yielded no colour reaction. This lead to suspecting the preservability of biotinylated antibodies. Biotinylated antibody made in January 2017 (170105MaK01) was tested to see how long the reagent would work. Test was done in September the same year and gave no results (Table 4).

Table 4. Biotinylated antibody tested using ricin concentration of 755 pg/ml.

	OD450				Mean
Blank	0.1051	0.0985	0.1039	0.0994	0.1017
755 pg/ml	0.0880	0.1035	0.0775	0.1026	0.0929

Biotinylated antibody stored in refrigerator at +4 ºC did not last nine months.

10.2 ELISA Protocol Optimization

Initial analysis was done using a different sandwich ELISA protocol. This protocol was based on the one used by Robert Koch Institute, described in reference 3: Characterization of Ricin and *R. communis* Agglutinin Reference Materials. Protocol was tested multiple times with no colour change after TMB incubation. After successful biotinylation first results were obtained (Table 5).

Table 5. First visible absorbance results.

Ricin (µg/ml)	OD450
Blank	0.0491
	0.0546
5.1	0.1226
	0.1205
	0.1273
	0.1308
	0.1136
	0.1167

Absorption results were very low, comparing to the concentration of ricin 5.1 µg/ml.

10.2.1 Blocking Buffer Optimization

Because of the low absorption values attention was directed towards possible disturbing factors. These factors were blocking buffer, biotinylation and coating buffer. Blocking buffer was changed from casein to 1 % BSA in PBS. Results in Table 6 were higher than using casein as blocking buffer.

Table 6. Casein blocker replaced with 1 % BSA in PBS OD450 results.

	mAb B c (µg/ml)			Mean mAb B c (µg/ml)		
Ricin (µg/ml)	2.5	10	20	2.5	10	20
Blank	0.0517	0.0532	0.0534	0.0516	0.0625	0.0563
	0.0514	0.0718	0.0592			
0.051	0.1719	0.1300	0.1332	0.1488	0.1323	0.1417
	0.1431	0.1389	0.1536			
	0.1313	0.1281	0.1383			
5.1	0.2045	0.2138	0.2154	0.2129	0.2056	0.2088
	0.2128	0.1925	0.2119			
	0.2215	0.2106	0.1990			

In blocking buffer test the concentration of coating antibody was tested also. Coating concentration 2.5 µg/ml gave highest results of three concentrations tested.

Blocking buffer change did not give adequate absorption results. Coating buffer change were considered. Coating was done using antibody diluted in PBS. Multiple other ELISA methods use alkaline coating buffer.

10.2.2 ELISA Protocol Change

A protocol used by Spiez laboratory in Switzerland had alkaline coating buffer, 1 % BSA in PBS as blocking buffer. Spiez protocol had also longer incubation times and incubation was done in higher temperatures than previously used protocol from RKI. Protocol was tested and the results were similar to blocking buffer change results (Table 7).

Table 7. Results after switching the protocol from RKI to Spiez

Ricin (µg/ml)	mAb B (µg/ml)			
	2.5	Mean	10	Mean
Blank	0.0542	0.0523	0.0499	0.0541
	0.0511		0.0575	
	0.0516		0.0548	
0.0051	0.1646	0.1991	0.1845	0.1764
	0.2540		0.1889	
	0.1786		0.1558	
0.051	0.2056	0.2676	0.3185	0.3311
	0.2293		0.3685	
	0.3679		0.3062	
0.51	0.2771	0.2619	0.2720	0.2651
	0.2681		0.2919	
	0.2405		0.2313	
5.1	0.2609	0.2620	0.2962	0.2919
	0.2513		0.3021	
	0.2737		0.2773	

A new biotinylation of antibodies was made using EZ-Link Sulfo-NHS-LC-Biotin and protocol was tested again. Results on Table 8 were significantly better with higher absorption reads.

Table 8. Results after EZ-Link Sulfo-NHS-LC-Biotin biotinylation with new protocol

Ricin (µg/ml)	OD450	Mean OD450
0	0.0454	0.0477
	0.0505	
	0.0473	
0.000755	0.4785	0.5004
	0.5268	
	0.4959	
0.00755	1.2015	1.4964
	1.4995	
	1.7882	
0.0755	1.9507	1.7812
	1.8581	
	1.5347	
0.755	1.7893	1.8124
	2.0155	
	1.6323	
7.55	2.1167	1.9654
	1.8545	
	1.9249	

10.2.3 Antibody Comparison and Concentration Optimization

Before testing, antibody protein concentration was measured with Lowry assay.

While developing ELISA method another antibody pair was tested. Antibody pair was HyTest RA999 and RB999. HyTest antibodies were used slightly differently from RKI antibodies: coating was done using RA999 against A chain and detection with EZ-Link Sulfo-NHS-LC-Biotin biotinylated RB999 against B chain. This was done because of HyTest recommendations. Results can be seen in Figure 8 and Appendix 1. RKI antibodies detected lower ricin concentrations than HyTest antibodies which could measure ricin from 0.76 ng/ml onwards.

Chosen antibody pair was RKI R109 and R18 biotinylated with EZ-Link Sulfo-NHS-LC-Biotin. Earlier R109 concentration was optimized to 2.5 µg/ml (Table 6, Table 7, and Appendix 1). Results for detection antibody optimization measurements are in Appendix 2. Optimum detection antibody concentration was 0.8 µg/ml. This concentration gave high absorption reads compared to other tested antibody concentrations.

Optimized antibody concentrations were for coating antibody R109 2.5 µg/ml and for detection antibody R18 0.8 µg/ml.

10.2.4 Conjugation Enzyme, poly-HRP, Optimization

Conjugation enzyme optimization results are found in Appendix 3. Concentration 1 ng/ml of poly-HRP improved detection of the lowest ricin standard 7.55 pg/ml and had higher absorption results throughout all standards. Poly-HRP concentration 1 ng/ml was chosen for optimized assay.

10.3 Validation

For validation calculations, a free program called MyAssays [38], was used for four parameter logistic curve fitting. With fitted curves absorption values were modified to concentration values.

Four parameter logistic (4PL) regression uses equation:

$$y = d + \frac{a-d}{1+(\frac{x}{c})^b} \quad (1)$$

where
x is independent variable
y is the dependent variable
a is the minimum value possible to obtained
d is the maximum value possible to obtained
c is the point of inflection, halfway between a and d
b is slope of the curve. [38]

To solve x from the equation [38]:

$$x = c(\frac{a-d}{y-d} - 1)^{\frac{1}{b}} \quad (2)$$

These calculations were done by the MyAssays program.

10.3.1 Limit of Blank and Limit of Detection

Limit of blank was calculated using multiple blank concentration results found in Table 9. Blank concentrations were calculated from results found in Appendix 4.

Table 9. Calculated concentration, average and standard deviation for blanks.

Blank c (pg/ml)			
0.287	1.914	1.810	0.754
1.060	0.968	1.686	1.645
1.851	0.685	2.432	1.117
0.618	0.842	0.914	1.117
0.987	1.060	0.932	
0.301	0.772	0.842	
0.568	1.385	1.173	
3.423	1.173	2.321	
2.610	0.454	0.987	
0.914	1.230	0.772	
Mean		1.224	
Standard deviation		0.695	
CV %		57	

Equation used for the calculations was.

$$LOB = mean_{blank} + 1.645 \times SD_{blank} \quad (3)$$

where

$mean_{blank}$ is the average result of all measured blanks

SD_{blank} is standard deviation of blanks [35].

Limit of detection was calculated using the equation

$$LOD = LOB + 1.645 \times SD_{low\ concentration\ sample} \quad (4)$$

where

$SD_{low\ concentration\ sample}$ is standard deviation result of lowest measured concentration [35].

LOD was calculated using the lowest ricin sample 2.5 pg/ml (Table 10.). Results used for the concentration calculations are found in Appendix 5.

Table 10. Three low ricin concentration results.

Ricin pg/ml	2.5	4	7.55
c pg/ml	2.906	3.861	8.596
	2.723	3.738	8.167
	2.814	4.035	8.106
	2.062	3.665	7.241
	2.520	3.787	7.447
	2.234	3.641	7.418
	2.723	4.671	6.125
Mean pg/ml	2.569	3.914	7.586
SD	0.314	0.359	0.809
CV %	12	9.2	11

Calculated LOB was 2.4 pg/ml and LOD was 2.9 pg/ml.

This meant that highest analyte concentration predicted to be found for this sandwich ELISA when sample contains no analyte was 2.4 pg/ml. Minimum detectable concentration for this sandwich ELISA was 2.9 pg/ml.

10.3.2 Intra-Assay and Inter-Assay Precision

Intra-assay precision 12 different ricin standards and all standards had eight duplicate standards. Results are found in Appendix 6 and calculated results in Table 11.

Table 11. Intra-assay precision results.

pg/ml		Mean (pg/ml)	SD	CV %
0	Sample 0	5.31	0.46	8.6
3.75	Sample 1	7.07	0.72	10
7.55	Sample 2	9.96	1.14	11
15	Sample 3	13.80	0.54	3.9
37.5	Sample 4	37.72	8.51	23
75.5	Sample 5	72.76	14.66	20
375	Sample 6	271.48	14.27	5.3
755	Sample 7	895.04	96.67	11
3750	Sample 8	5179.13	2591.06	50
7550	Sample 9	10733.75	11241.15	105
75500	Sample 10	12118.88	2424.34	20
375000	Sample 11	12510.00	2913.28	23

Results were relatively good. Half of the CV % results were close or below 10 % and the other half around 20 %. Only results for samples 8 and 9 were inferior. This could be because of a pipetting error. Samples 8 and 9 are also on the angle of the curve were saturation begins. Samples 10 and 11 are on the plain part of the curve and results thus closer to ideal. One reason for high CV % can be that the logarithmic scale can make even small differences between OD450 values larger.

Inter-assay precision had 17 determinations during 4 different days. Results can be found in Appendix 7 and calculated results in Table 12.

Table 12. Inter-assay precision results.

Ricin (pg/ml)	7.55	35	75.5	755	7550
pg/ml	6.9	37.2	67.8	784.7	3389.0
	5.9	38.7	74.1	638.1	6376.0
	5.9	36.4	79.9	872.7	> Curve
	6.9	38.3	71.7	1372.0	3133.0
	7.2	39.7	66.7	692.0	3071.0
	6.5	38.5	75.6	555.5	> Curve
	4.7	38.6	105.4	699.9	6706.0
	7.7	36.7	83.3	739.3	> Curve
	5.7	34.2	69.0	716.0	4355.0
	8.5	34.1	68.6	724.1	4389.0
	5.3	59.7	68.4	935.1	4457.0
	4.6	33.9	66.2	704.5	> Curve
	6.1	33.5	63.3	749.1	4953.0
	7.6	34.4	66.0	798.7	4395.0
	7.1	34.7	82.3	696.5	3413.0
	11.0	36.8	71.3	638.3	8455.0
	6.7	36.2	70.2	987.4	> Curve
Mean pg/ml	6.7	37.7	73.5	782.6	4757.7
SD	1.5	6.0	10.1	186.0	1646.2
CV %	23	16	14	24	35

All inter-assay precision results were over the preferred 10 % limit. This shows that replicability of the assay is not very good. Especially the higher concentrations gave worse results indicating that saturation hampers the assay precision. Result marked as > Curve means that the value is greater than upper asymptote of the four parameter logistic.

10.3.3 Measurement from Spiked Matrices

Average recovery % results for three different matrices, milk, wine and flour can be found in Table 13, 14, 15 and 16. Results were calculated using fitted curves and concentrations found in Appendix 8.

Table 13. Ricin spiked on milk recovery %.

Sample		Average recovery %	Sample		Average recovery %
MILK, diluted with milk matrix	1	21	MILK, diluted with 0.03 % BSA 0.1% TritonX in PBS		
	2	8.5		2	26
	3	15		3	15
	4	85		4	9.6
	5	450		5	5.4
	6	6.3		6	1.3
	7	0.5		7	0.1

Two different dilutions gave somewhat different results. When diluting the spiked milk with milk it seems to have slightly higher results than diluting with 0.03 % BSA 0.1% TritonX in PBS. It also has recovery of 450 % hinting that the method is not exact for different matrices.

Table 14. Ricin spiked wine recovery %.

Sample		Average recovery %	Sample		Average recovery %
WINE, diluted with wine matrix	1	18	WINE, diluted with 0.03 % BSA 0.1% TritonX in PBS		
	2	3.0		2	11
	3	0.22		3	14
	4	0.03		4	10
	5	0.09		5	8.2
	6	0.00		6	3.6
	7	0.05		7	0.2

With wine matrix results seem to be opposite from milk results. Diluting the spiked wine with 0.03 % BSA 0.1% TritonX in PBS seems to improve recovery %. This might indicate that wine has something that hinders assay and when diluted with optimum solution that interference diminishes.

Table 15. Ricin spiked pH changed wine recovery %

Sample		Average recovery %
WINE pH 8		
	2	0.04
	3	1.92
	4	0.02
	6	0.02
	7	0.06

Changing the pH of the wine seems not to increase recovery, so assay hindering factor is probably not pH level of the matrix.

Table 16. Ricin spiked flour recovery %.

Sample		Average recovery %	Sample		Average recovery %
FLOUR, extraction with 2 % acetic acid	1	22	FLOUR, extraction with PBST	1	25
	2	7.0		2	1.9
	3	0.29		3	0.1
	4	0.03		4	0.4
	5	0.01		5	0.3
	6	0.003		6	0.2
	7	0.01		7	0.1

Flour extraction with PBST gives overall better recovery % than with 2 % acetic acid. More neutral extraction solution improves the recovery of ricin.

Flour matrix seems to be difficult matrix to detect ricin compared to other two matrices. The other two yield higher recoveries, but milk appears to be the easiest to detect ricin from.

Ricin could be enriched from the spiked samples using for example antibody coated magnetic beads, to enhance recovery % from the different matrices.

10.3.4 Specificity

Results for ricin sandwich ELISA cross-reactivity with agglutinin can be found in Table 17. Absorbance of concentrations between 1-10 ng/ml do not differ from blank. First detectable agglutinin concentration is 25 ng/ml.

Table 17. Measured OD450 values for agglutinin RCA120 when using ricin sandwich ELISA.

Agglutinin (ng/ml)	OD450			Mean
0	0.0580	0.0573	0.0566	0.0573
1	0.0536	0.0563	0.0553	0.0551
5	0.0564	0.0610	0.0606	0.0593
10	0.0581	0.0566	0.0579	0.0575
25	0.0742	0.0955	0.0615	0.0771
50	0.0802	0.0718	0.0664	0.0728
100	0.0671	0.0695	0.0834	0.0733
1000	0.1689	0.1404	0.1631	0.1575

Abrin results (Table 18) show that all measured concentrations were same as blank. Abrin used in the test was not purified and may contain other proteins.

Table 18. Measured OD450 values for abrin when using ricin ELISA.

Abrin (ng/ml)	OD450			Mean
0	0.0921	0.0982	0.1045	0.0983
1	0.1012	0.0923	0.0949	0.0961
5	0.0933	0.0913	0.0869	0.0905
10	0.0899	0.0910	0.0928	0.0912
20	0.0898	0.0907	0.0909	0.0905
40	0.0893	0.0857	0.0988	0.0913
400	0.0853	0.0894	0.0929	0.0892
4000	0.0818	0.0913	0.0864	0.0865

Agglutinin 1–1000 ng/ml and abrin 1–4000 ng/ml were assayed with ricin sandwich ELISA. Abrin was not cross-reacting with the developed ricin ELISA. Agglutinin was found to have some cross-reactivity when concentration of agglutinin was 25 ng/ml or higher.

11 Discussion and Conclusion

This study concentrated on developing enzymatic methods of ricin determination. Particularly on method optimization and validation of sandwich ELISA. Purpose was to improve existing method to attain reliable results in ricin determination and provide VERIFIN with detailed working instruction. The optimization work concentrated on antibody biotinylation, reaction buffers, antibody concentrations and conjugate enzyme concentration. After optimizing the method, the focus was on method validation.

Validation focused on limit of blank, limit of detection, assay precision, recovery and specificity.

Method optimization was successful and developed assay is capable of detecting ricin easily between concentrations 7.6 pg/ml–7.6 µg/ml when using standard dilution solution 0.03 % BSA 0.1 % TritonX in PBS. Suitable buffer for coating was found to be carbonate bicarbonate buffer and for blocking 1 % BSA in PBS. Best tested concentrations for antibodies were 2.5 µg/ml for capture antibody and 0.8 µg/ml for detection antibody. Conjugate enzyme poly-HRP concentration was most suitable at 1 ng/ml. Optimal biotin for antibody labelling was EZ-Link Sulfo-NHS-LC-Biotin.

Limit of detection was 2.9 pg/ml. Intra-assay precision varied from 3.9 % to 105 %. Inter-assay precision was between 14 % and 35 %. Precisions for both were higher than 10 % which indicates that the replicability of the assay is not reliable in current work. Precision could be positively influenced by washing microplates using plate washer rather than washing with pipetting by hand, and possible changing to electronic pipette.

Measurement with spiked matrices from milk, wine and flour yielded low results. Milk gave the best results varying between 0.1–450 %. Because recovery was not between 80 % and 120 % it can be considered that analysed matrices had substantial effect on the ELISA assay, and, thus quantification of the ricin. To improve recovery % sample containing ricin could be concentrated and then diluted using PBST or analyte could be enriched with antibodies.

No abrin cross-reactivity was observed with the assay and agglutinin reacted with the ricin antibodies when concentration of it reached 25 ng/ml. This indicates that used antibodies were highly specific for ricin identification. When considering all validation results the assay did not yield repeatable accurate results in the current work.

The goal of the thesis was met providing VERIFIN with working sandwich ELISA method detecting ricin in low concentrations. Even though validation was not optimal, with some changes it can be improved, and the optimized ELISA method will yield precise results.

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RKI and HyTest antibody comparison results

Ricin	Coating mAb	Concentratio (ng/ml)	ODT450			Mean
B3249	RKI 2,5 µg/ml	0	0.0503	0.0384	0.0392	0.0388
		0.00755	0.0398	0.0368	0.0483	0.0416
		0.0755	0.0666	0.0701	0.0587	0.0684
		0.755	0.2473	0.2428	0.2619	0.2524
		7.55	0.5629	0.4430	0.5232	0.5431
		75.5	0.1187	0.4948	0.4914	0.4931
B3249	RKI 5 µg/ml	0	0.0393	0.0411	0.1965	0.0402
		0.00755	0.0356	0.0346	0.0449	0.0384
		0.0755	0.0353	0.0735	0.0757	0.0746
		0.755	0.2047	0.2583	0.2458	0.2521
		7.55	0.4389	0.5072	0.4492	0.4441
		75.5	0.4962	0.3751	0.5314	0.5138
B2578	RKI 2,5 µg/ml	0	0.0494	0.0459	0.0447	0.0467
		0.0051	0.0475	0.0474	0.0470	0.0473
		0.051	0.0636	0.0606	0.0570	0.0604
		0.51	0.1664	0.1706	0.1736	0.1702
		5.1	0.5883	0.4276	0.4579	0.4428
		51	0.5008	0.5037	0.4852	0.4930

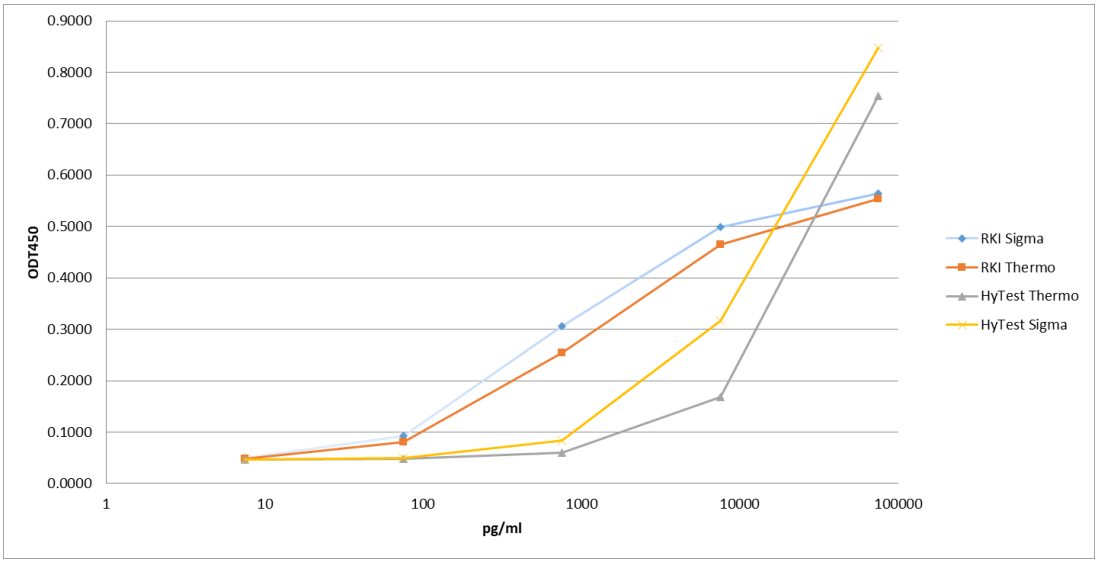
Ricin	Coating mAb	Concentratio (ng/ml)	ODT450		Mean
B3249	HyTest 2,5 µg/ml	0	0.0391	0.0367	0.0379
		0.00755	0.0347	0.0342	0.0345
		0.0755	0.0349	0.0343	0.0346
		0.755	0.0453	0.0626	0.0540
		7.55	0.1559	0.2131	0.2131
		75.5	0.4251	0.1582	0.4251

Ricin	Coating mAb	Detection mAb	Ricin concentration (ng/ml)	ODT450			Mean
B3249	RKI 2,5 µg/ml	RKI 1 µg/ml + Sulfo-NHS-LC-Biotin	0	0.0861	0.0468	0.0469	0.0469
			0.00755	0.0495	0.0467	0.0484	0.0482
			0.0755	0.0818	0.0794	0.1415	0.0806
			0.755	0.2553	0.2539	0.3020	0.2546
			7.55	0.4691	0.4645	0.4627	0.4654
			75.5	0.5570	0.7163	0.5509	0.5540

Ricin	Coating mAb	Detection mAb	Ricin concentration (ng/ml)	ODT450			keskiarvo
B3249	RKI 2,5 µg/ml	RKI 1 µg/ml + Biotinamidohexanoyl-6-aminohexanoic acid NHS ester	0	0.0478	0.0531	0.0460	0.0490
			0.00755	0.0483	0.0505	0.0493	0.0494
			0.0755	0.0923	0.0911	0.0923	0.0919
			0.755	0.2973	0.3192	0.2998	0.3054
			7.55	0.4975	0.4859	0.5127	0.4987
			75.5	0.7178	0.5666	0.5623	0.5645

Ricin	Coating mAb	Detection mAb	Ricin concentration (ng/ml)	ODT450			keskiarvo
B3249	HyTest 2,5 µg/ml	HyTest 1 µg/ml + Sulfo-NHS-LC-Biotin	0	0.0483	0.0508	0.0455	0.0482
			0.00755	0.0499	0.0446	0.0463	0.0469
			0.0755	0.0496	0.0477	0.0476	0.0483
			0.755	0.0602	0.0570	0.0605	0.0592
			7.55	0.1704	0.1976	0.1674	0.1689
			75.5	0.7497	0.4964	0.7578	0.7538

Ricin	Coating mAb	Detection mAb	Ricin concentration (ng/ml)	ODT450			keskiarvo
B3249	HyTest 2,5 µg/ml	HyTest 1 µg/ml + Biotinamidohexanoyl-6-aminohexanoic acid NHS ester	0	0.0463	0.0462	0.0469	0.0465
			0.00755	0.0473	0.0446	0.0484	0.0468
			0.0755	0.0485	0.0501	0.0500	0.0495
			0.755	0.0815	0.0887	0.0807	0.0836
			7.55	0.2761	0.3013	0.3705	0.3160
			75.5	0.8825	0.8123	0.6743	0.8474



Detection antibody optimization results

Detection mAb c (µg/ml)	Ricin (ng/ml)	OD450		Mean
0.2	0	0.0665	0.0512	0.05885
	0.00755	0.0496	0.0494	0.0495
	0.0755	0.0612	0.0749	0.06805
	0.755	0.6705	0.2518	0.4612
	7.55	0.6163	0.7564	0.68635
	75.5	0.6740	0.6216	0.6478

Detection mAb c (µg/ml)	Ricin (ng/ml)	OD450		Mean
0.3	0	0.0516	0.0457	0.04865
	0.00755	0.0502	0.0457	0.04795
	0.0755	0.0673	0.0834	0.07535
	0.755	0.2835	0.4024	0.3430
	7.55	0.5005	0.4952	0.49785
	75.5	0.6194	0.6329	0.6262

Detection mAb c (µg/ml)	Ricin (ng/ml)	OD450		Mean
0.4	0	0.0519	0.0529	0.0524
	0.00755	0.0492	0.0492	0.0492
	0.0755	0.0603	0.0647	0.0625
	0.755	0.2850	0.2404	0.2627
	7.55	0.6309	0.4896	0.56025
	75.5	0.7926	0.6205	0.7066

Detection mAb c (µg/ml)	Ricin (ng/ml)	OD450		Mean
0.5	0	0.0441	0.0528	0.04845
	0.00755	0.0476	0.0504	0.049
	0.0755	0.0685	0.0686	0.06855
	0.755	0.2552	0.2483	0.2518
	7.55	0.5091	0.5427	0.5259
	75.5	0.6207	0.6111	0.6159

Detection mAb c (µg/ml)	Ricin (ng/ml)	OD450		Mean
0.6	0	0.0469	0.0444	0.04565
	0.00755	0.0478	0.0460	0.0469
	0.0755	0.0716	0.0678	0.0697
	0.755	0.3006	0.2814	0.2910
	7,55	0,5283	0,6703	0,5993
	75,5	0,6081	0,6824	0,6453

Detection mAb c (μg/ml)	Ricin (ng/ml)	OD450		Mean
0.7	0	0.0441	0.0440	0.04405
	0.00755	0.0447	0.0453	0.045
	0.0755	0.0688	0.0623	0.06555
	0.755	0.2573	0.2567	0.2570
	7.55	0.7054	0.6261	0.66575
	75.5	0.8127	0.5706	0.6917

Detection mAb c (μg/ml)	Ricin (ng/ml)	OD450		Mean
0.8	0	0.0451	0.0437	0.0444
	0.00755	0.0474	0.0467	0.04705
	0.0755	0.0715	0.0717	0.0716
	0.755	0.3721	0.2931	0.3326
	7.55	0.7862	0.7982	0.7922
	75.5	0.7505	0.7815	0.7660

Detection mAb c (μg/ml)	Ricin (ng/ml)	OD450		Mean
0.9	0	0.0628	0.052	0.0574
	0.00755	0.0501	0.0505	0.0503
	0.0755	0.0719	0.0620	0.06695
	0.755	0.2184	0.2204	0.2194
	7.55	0.4728	0.4508	0.4618
	75.5	0.5787	0.5818	0.5803

Detection mAb c (μg/ml)	Ricin (ng/ml)	OD450		Mean
1	0	0.0460	0.0503	0.04815
	0.00755	0.0496	0.0471	0.04835
	0.0755	0.0692	0.0658	0.0675
	0.755	0.2142	0.2158	0.2150
	7.55	0.4388	0.5419	0.49035
	75.5	0.5778	0.5826	0.5802

Detection mAb c (μg/ml)	Ricin (ng/ml)	OD450		Mean
1.1	0	0.0482	0.0539	0.05105
	0.00755	0.0486	0.0469	0.04775
	0.0755	0.0755	0.0691	0.0723
	0.755	0.2079	0.2102	0.2091
	7.55	0.4475	0.4626	0.45505
	75.5	0.5641	0.5749	0.5695

Detection mAb c (μg/ml)	Ricin (ng/ml)	OD450		Mean
1.2	0	0.0443	0.0461	0.0452
	0.00755	0.0479	0.0523	0.0501
	0.0755	0.0673	0.0690	0.06815
	0.755	0.2699	0.2160	0.2430
	7.55	0.4680	0.4805	0.47425
	75.5	0.6749	0.5724	0.6237

Detection mAb c (μg/ml)	Ricin (ng/ml)	OD450		Mean
1.3	0	0.0489	0.0487	0.0488
	0.00755	0.0488	0.0483	0.04855
	0.0755	0.0694	0.0677	0.06855
	0.755	0.2411	0.2208	0.2310
	7.55	0.5960	0.5762	0.5861
	75.5	0.5572	0.5242	0.5407

Detection mAb c (μg/ml)	Ricin (ng/ml)	OD450		Mean
1.4	0	0.0542	0.0526	0.0534
	0.00755	0.0504	0.0485	0.04945
	0.0755	0.0685	0.0675	0.068
	0.755	0.2089	0.1901	0.1995
	7.55	0.5443	0.6097	0.577
	75.5	0.5056	0.5330	0.5193

Detection mAb c (μg/ml)	Ricin (ng/ml)	OD450		Mean
1.5	0	0.0485	0.0488	0.04865
	0.00755	0.0497	0.0476	0.04865
	0.0755	0.0641	0.0643	0.0642
	0.755	0.1712	0.1916	0.1814
	7.55	0.4153	0.4712	0.44325
	75.5	0.6444	0.5017	0.5731

Poly-HRP optimization results

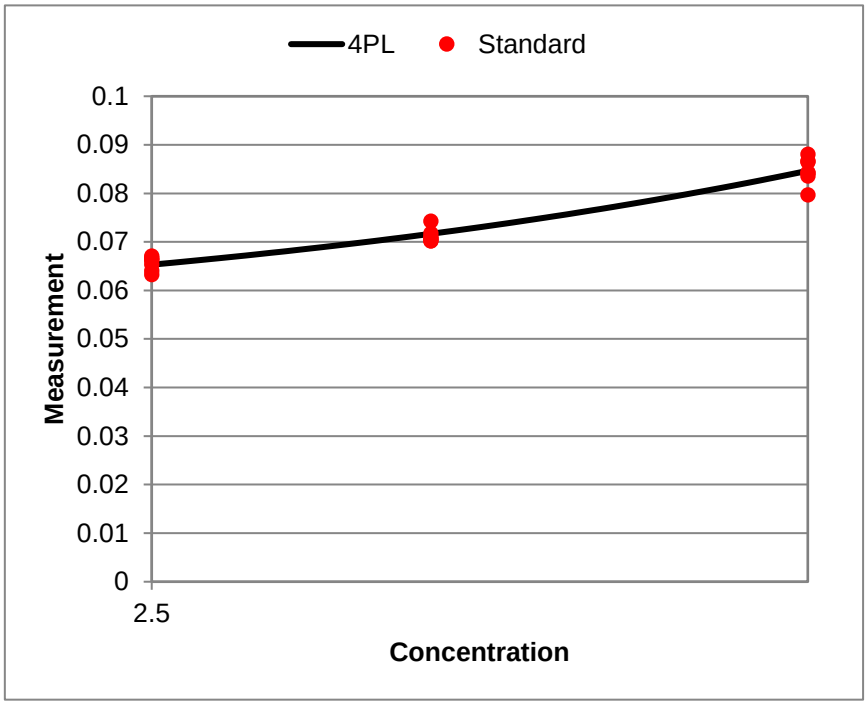
poly-HRP c (ng/ml)	Ricin (ng/ml)	ODT450			Mean
0.25	0	0.0463	0.0476	0.0481	0.047333
	0.00755	0.0472	0.0503	0.0490	0.0488
	0.02	0.0513	0.0563	0.0494	0.0523
	0.0755	0.0626	0.0706	0.0648	0.0637
	0.755	0.1776	0.2217	0.1756	0.1766
	7.55	0.3012	0.4051	0.3997	0.4024
	75.5	0.5048	0.4962	0.4832	0.4947

poly-HRP c (ng/ml)	Ricin (ng/ml)	ODT450			Mean
0.5	0	0.0488	0.0502	0.0503	0.0498
	0.00755	0.0509	0.0662	0.0517	0.0513
	0.02	0.0570	0.0586	0.0597	0.0584
	0.0755	0.0849	0.0862	0.0914	0.0856
	0.755	0.3428	0.3290	0.3407	0.3375
	7.55	0.7637	0.7662	1.0033	0.7650
	75.5	0.9354	0.9554	0.9290	0.9399

poly-HRP c (ng/ml)	Ricin (ng/ml)	ODT450			Mean
0.75	0	0.0517	0.0500	0.0505	0.0507
	0.00755	0.0542	0.0565	0.0546	0.0551
	0.02	0.0636	0.0678	0.0621	0.0645
	0.0755	0.1099	0.1109	0.1113	0.1107
	0.755	0.4802	0.5176	0.4687	0.4745
	7.55	1.1893	1.1349	1.1530	1.1440
	75.5	2.2191	1.3727	1.6219	1.4973

poly-HRP c (ng/ml)	Ricin (ng/ml)	ODT450			Mean
1	0	0.0484	0.0502	0.0537	0.0508
	0.00755	0.0530	0.0547	0.0553	0.0543
	0.02	0.0657	0.0654	0.0757	0.0656
	0.0755	0.1681	0.1268	0.2771	0.1475
	0.755	0.6583	0.5798	0.5980	0.5889
	7.55	1.3249	1.3282	1.3683	1.3405
	75.5	2.6349	1.5819	1.6129	1.5974

MyAssays LOB results



a	0.05132
b	0.8311
c	138.2
d	0.458
MSE	0.00001098
R^2	0.9311
SS	0.0003185
SYX	0.003569

Calibrator	Wells	Conc.	Raw	SEM	Backfit	Recovery %
Standard1	A1	0	0.0458	0.0021	< Curve	-
	B1		0.0456		< Curve	-
	C1		0.0487		< Curve	-
	D1		0.049		< Curve	-
	E1		0.0498		< Curve	-
	F1		0.0508		< Curve	-
	G1		0.0583		1.06	-
	H1		0.0623		1.851	-
Standard2	A2	2.5	0.0671	0.000535	2.906	116.2
	B2		0.0663		2.723	108.9
	C2		0.0667		2.814	112.6
	D2		0.0633		2.062	82.48
	E2		0.0654		2.52	100.8
	F2		0.0641		2.234	89.37
	G2		Flagged		-	-
	H2		0.0663		2.723	108.9
Standard3	A3	4	Flagged	0.00054	-	-
	B3		0.0711		3.861	96.53

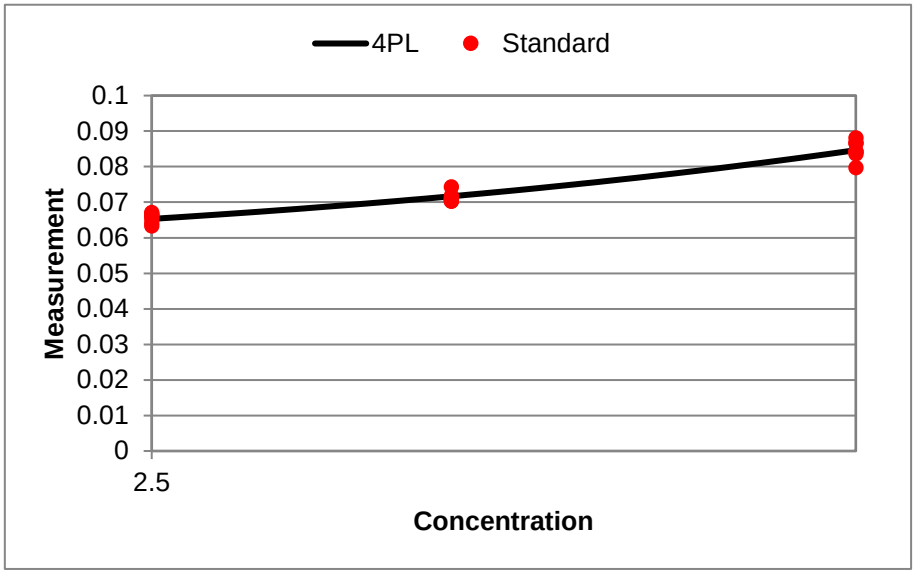
	C3		0.0706		3.738	93.46
	D3		0.0718		4.035	100.9
	E3		0.0703		3.665	91.62
	F3		0.0708		3.787	94.68
	G3		0.0702		3.641	91.01
	H3		0.0743		4.671	116.8
Standard4	A4	7.55	0.0881	0.00104	8.596	113.9
	B4		0.0867		8.167	108.2
	C4		0.0865		8.106	107.4
	D4		0.0836		7.241	95.91
	E4		0.0843		7.447	98.64
	F4		0.0842		7.418	98.25
	G4		0.0797		6.125	81.12
	H4		Flagged		-	-

Sample	Wells	Raw	Conc.	Conc. (Average)	%CV	SD	SEM
Unknown1	A5	0.0451	< Curve	-	-	-	-
Unknown2	B5	0.0437	< Curve	-	-	-	-
Unknown3	C5	0.0484	< Curve	-	-	-	-
Unknown4	D5	0.0502	< Curve	-	-	-	-
Unknown5	E5	0.0537	0.2868	0.2868	-	-	0
Unknown6	F5	0.0474	< Curve	-	-	-	-
Unknown7	G5	0.0474	< Curve	-	-	-	-
Unknown8	H5	0.0466	< Curve	-	-	-	-
Unknown9	A6	0.0458	< Curve	-	-	-	-
Unknown10	B6	0.0456	< Curve	-	-	-	-
Unknown11	C6	0.0487	< Curve	-	-	-	-
Unknown12	D6	0.049	< Curve	-	-	-	-
Unknown13	E6	0.0498	< Curve	-	-	-	-
Unknown14	F6	0.0508	< Curve	-	-	-	-
Unknown15	G6	0.0583	1.06	1.06	-	-	0
Unknown16	H6	0.0623	1.851	1.851	-	-	0
Unknown17	A7	0.0558	0.6175	0.6175	-	-	0
Unknown18	B7	0.0579	0.9866	0.9866	-	-	0
Unknown19	C7	0.0538	0.3014	0.3014	-	-	0
Unknown20	D7	0.0555	0.5676	0.5676	-	-	0
Unknown21	E7	0.0693	3.423	3.423	-	-	0
Unknown22	F7	0.0658	2.61	2.61	-	-	0
Unknown23	G7	0.0575	0.9138	0.9138	-	-	0
Unknown24	H7	0.0626	1.914	1.914	-	-	0

Unknown25	A8	0.0578	0.9683	0.9683	-	-	0
Unknown26	B8	0.0562	0.6852	0.6852	-	-	0
Unknown27	C8	0.0571	0.8422	0.8422	-	-	0
Unknown28	D8	0.0583	1.06	1.06	-	-	0
Unknown29	E8	0.0567	0.7716	0.7716	-	-	0
Unknown30	F8	0.06	1.385	1.385	-	-	0
Unknown31	G8	0.0589	1.173	1.173	-	-	0
Unknown32	H8	0.0548	0.4544	0.4544	-	-	0
Unknown33	A9	0.0592	1.23	1.23	-	-	0
Unknown34	B9	0.0621	1.81	1.81	-	-	0
Unknown35	C9	0.0615	1.686	1.686	-	-	0
Unknown36	D9	0.065	2.432	2.432	-	-	0
Unknown37	E9	0.0575	0.9138	0.9138	-	-	0
Unknown38	F9	0.0576	0.9319	0.9319	-	-	0
Unknown39	G9	0.0571	0.8422	0.8422	-	-	0
Unknown40	H9	0.0589	1.173	1.173	-	-	0
Unknown41	A10	0.0463	< Curve	-	-	-	-
Unknown42	B10	0.0461	< Curve	-	-	-	-
Unknown43	C10	0.0645	2.321	2.321	-	-	0
Unknown44	D10	0.0579	0.9866	0.9866	-	-	0
Unknown45	E10	0.0567	0.7716	0.7716	-	-	0
Unknown46	F10	0.0566	0.7542	0.7542	-	-	0
Unknown47	G10	0.0613	1.645	1.645	-	-	0
Unknown48	H10	0.0586	1.117	1.117	-	-	0
Unknown49	A11	0.0586	1.117	1.117	-	-	0

The highlighted samples are outside the range of the curve fit and/or the range of the standards.

MyAssays LOD results

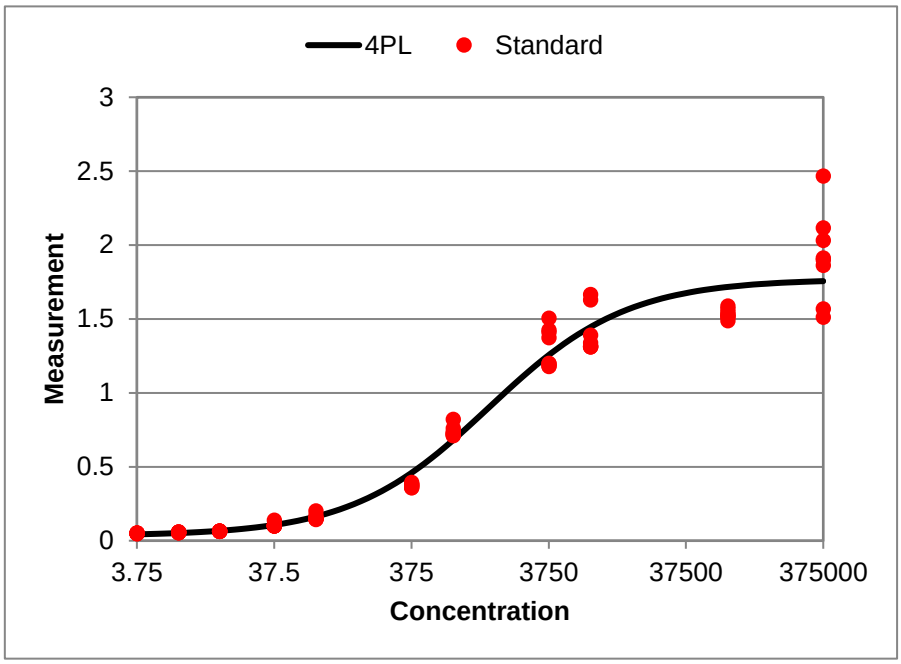


a	0.05132
b	0.8311
c	138.2
d	0.458
MSE	0.00001098
R^2	0.9311
SS	0.0003185
SYX	0.003569

Calibrator	Conc.	Wells	Raw	SEM	Backfit	Recovery %
Standard1	0	A1	0.0458	0.0021	< Curve	-
		B1	0.0456		< Curve	-
		C1	0.0487		< Curve	-
		D1	0.049		< Curve	-
		E1	0.0498		< Curve	-
		F1	0.0508		< Curve	-
		G1	0.0583		1.06	-
		H1	0.0623		1.851	-
Standard2	2.5	A2	0.0671	0.000535	2.906	116.2
		B2	0.0663		2.723	108.9
		C2	0.0667		2.814	112.6
		D2	0.0633		2.062	82.48
		E2	0.0654		2.52	100.8
		F2	0.0641		2.234	89.37
		G2	Flagged		-	-
		H2	0.0663		2.723	108.9
Standard3	4	A3	Flagged	0.00054	-	-
		B3	0.0711		3.861	96.53
		C3	0.0706		3.738	93.46

		D3	0.0718		4.035	100.9
		E3	0.0703		3.665	91.62
		F3	0.0708		3.787	94.68
		G3	0.0702		3.641	91.01
		H3	0.0743		4.671	116.8
Standard4	7.55	A4	0.0881	0.00104	8.596	113.9
		B4	0.0867		8.167	108.2
		C4	0.0865		8.106	107.4
		D4	0.0836		7.241	95.91
		E4	0.0843		7.447	98.64
		F4	0.0842		7.418	98.25
		G4	0.0797		6.125	81.12
		H4	Flagged		-	-

MyAssays intra-assay precision results



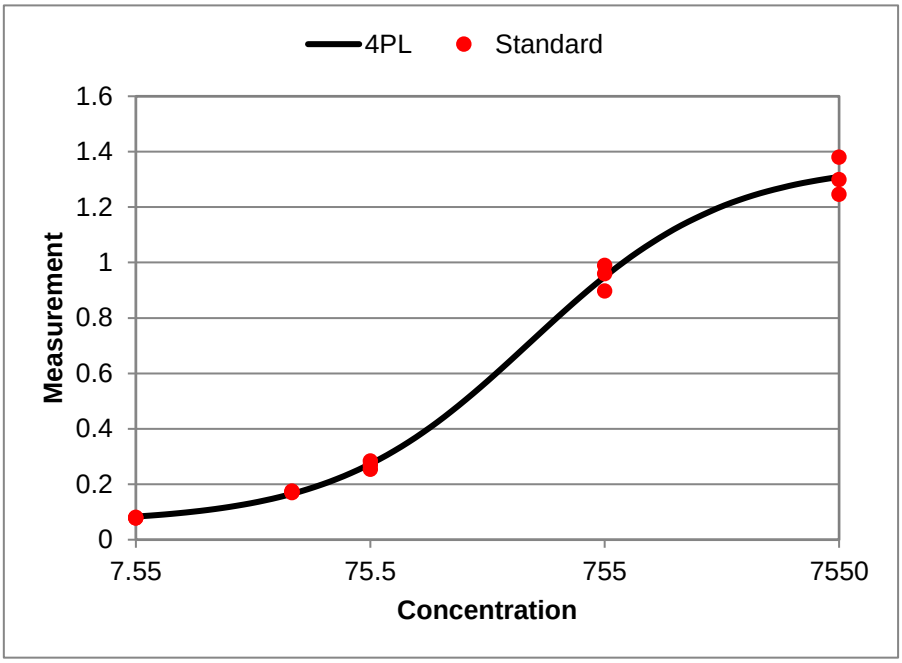
a	0.03263
b	0.8646
c	1376
d	1.769
MSE	0.01613
R^2	0.9653
SS	1.548
SYX	0.1297

Calibrator	Wells	Conc.	Raw	SEM	Backfit	Recovery %
Standard1	A1	0	0.0474	0.000366	5.6	-
	B1		0.0474		5.6	-
	C1		0.0466		5.248	-
	D1		0.0463		5.117	-
	E1		0.0461		5.029	-
	F1		0.0458		4.899	-
	G1		0.0456		4.812	-
	H1		0.0487		6.179	-
Standard2	A2	3.75	0.0522	0.00055	7.779	207.5
	B2		0.0536		8.435	224.9
	C2		0.0505		6.995	186.5
	D2		0.051		7.224	192.7
	E2		0.0491		6.359	169.6
	F2		0.0499		6.721	179.2
	G2		0.0493		6.45	172
	H2		0.0497		6.631	176.8

Standard3	A3	7.55	0.0543	0.000828	8.765	116.1
	B3		0.059		11.03	146.2
	C3		0.0558		9.48	125.6
	D3		0.0603		11.68	154.7
	E3		0.0553		9.241	122.4
	F3		0.0541		8.671	114.8
	G3		0.0565		9.817	130
	H3		0.0589		10.99	145.5
Standard4	A4	15	0.0653	0.000372	14.2	94.65
	B4		0.0653		14.2	94.65
	C4		0.0646		13.84	92.27
	D4		0.0633		13.18	87.86
	E4		0.0633		13.18	87.86
	F4		0.0635		13.28	88.54
	G4		0.0661		14.61	97.39
	H4		0.0647		13.89	92.61
Standard5	A5	37.5	0.107	0.0047	38.02	101.4
	B5		0.105		36.3	96.81
	C5		0.101		34	90.68
	D5		0.101		33.88	90.36
	E5		0.139		58.4	155.7
	F5		0.1		33.7	89.88
	G5		0.0996		33.34	88.92
	H5		0.101		34.12	91
Standard6	A6	75.5	0.203	0.00698	105.7	140
	B6		0.173		82.37	109.1
	C6		0.152		67.72	89.69
	D6		0.152		67.37	89.23
	E6		0.152		67.16	88.95
	F6		0.148		64.99	86.08
	G6		0.144		62.02	82.14
	H6		0.148		64.71	85.71
Standard7	A7	375	0.373	0.00441	269	71.72
	B7		0.395		294.9	78.63
	C7		0.377		273.9	73.03
	D7		0.382		279.2	74.44
	E7		0.385		283.1	75.5
	F7		0.358		252.4	67.3
	G7		0.368		263.3	70.21
	H7		0.362		256	68.28
Standard8	A8	755	0.711	0.013	822.5	108.9
	B8		0.732		871.5	115.4
	C8		0.72		842.5	111.6

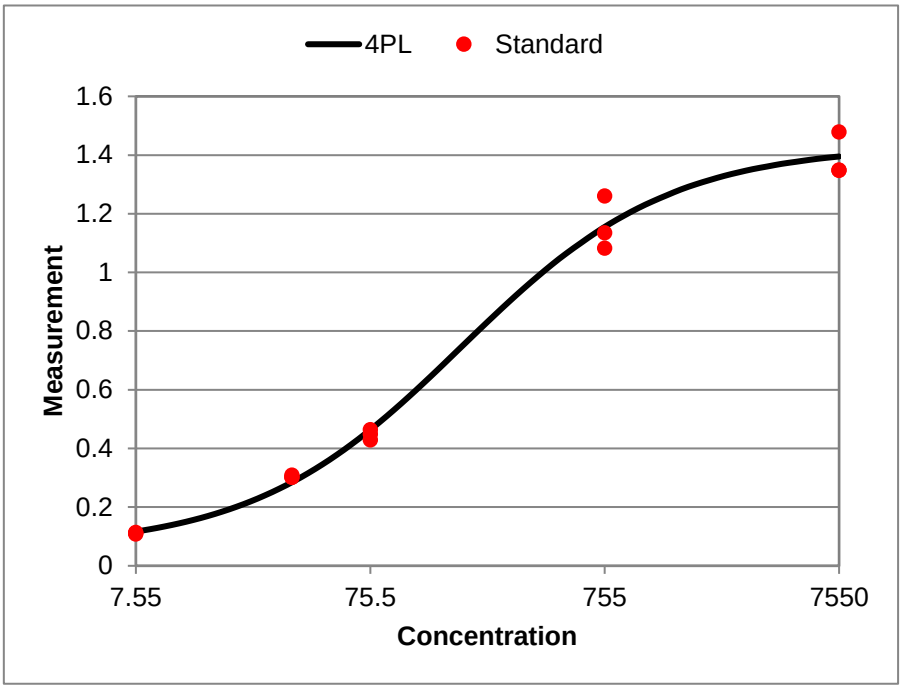
	D8		0.724		852.6	112.9
	E8		0.822		1114	147.5
	F8		0.714		828.3	109.7
	G8		0.737		883.7	117
	H8		0.761		945.2	125.2
Standard9	A9	3750	1.2	0.0469	3126	83.37
	B9		1.43		6927	184.7
	C9		1.18		3011	80.28
	D9		1.51		10090	269
	E9		1.41		6496	173.2
	F9		1.18		2966	79.1
	G9		1.37		5651	150.7
	H9		1.2		3166	84.43
Standard10	A10	7550	1.39	0.0528	6068	80.37
	B10		1.67		33720	446.6
	C10		1.31		4519	59.85
	D10		1.31		4545	60.2
	E10		1.32		4597	60.89
	F10		1.63		22910	303.4
	G10		1.34		4961	65.71
	H10		1.31		4550	60.26
Standard11	A11	75500	1.59	0.0115	16610	22
	B11		1.57		14590	19.33
	C11		1.55		12630	16.73
	D11		1.52		10600	14.04
	E11		1.53		11750	15.57
	F11		1.49		9221	12.21
	G11		1.51		10500	13.91
	H11		1.52		11050	14.64
Standard12	A12	375000	1.57	0.107	14570	3.884
	B12		1.9		> Curve	-
	C12		1.86		> Curve	-
	D12		2.47		> Curve	-
	E12		1.51		10450	2.787
	F12		2.03		> Curve	-
	G12		2.12		> Curve	-
	H12		1.91		> Curve	-

MyAssay inter-assay precision results



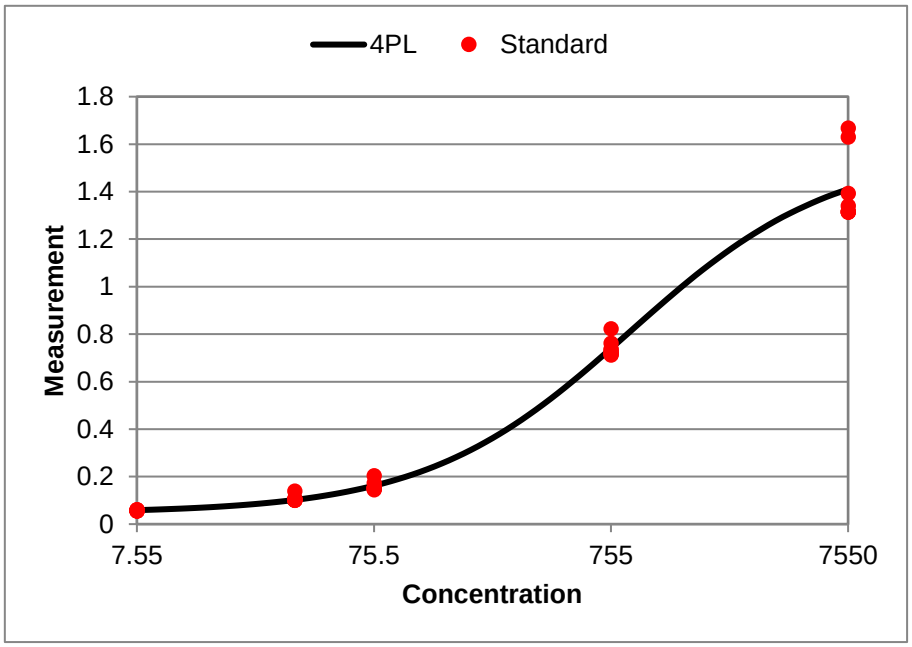
a	0.06088
b	1.042
c	362.4
d	1.362
MSE	0.0007965
R^2	0.9966
SS	0.01434
SYX	0.032

Calibrator	Wells	Conc.	Raw	SEM	Backfit	Recovery %
Standard1	A1	0	0.0583	0.00181	< Curve	-
	B1		0.0623		0.5203	-
	C1		0.0645		1.281	-
Standard2	A2	7.55	0.0814	0.00095	6.86	90.86
	B2		0.0785		5.914	78.33
	C2		0.0786		5.946	78.76
Standard3	A3	35	0.172	0.00196	37.2	106.3
	B3		0.176		38.71	110.6
	C3		0.17		36.36	103.9
Standard4	A4	75.5	0.254	0.00864	67.77	89.76
	B4		0.27		74.06	98.09
	C4		0.284		79.88	105.8
Standard5	A5	755	0.96	0.0272	784.7	103.9
	B5		0.898		638.1	84.51
	C5		0.99		872.7	115.6
Standard6	A6	7550	1.25	0.0391	3389	44.89
	B6		1.3		6376	84.45
	C6		1.38		> Curve	-



a	0.05874
b	0.9774
c	183.9
d	1.43
MSE	0.001721
R^2	0.9935
SS	0.03097
SYX	0.04704

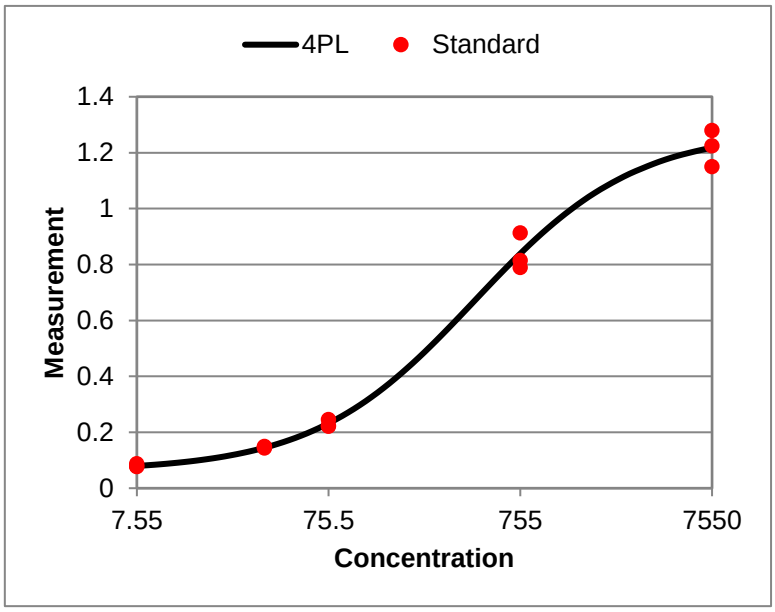
Calibrator	Wells	Conc.	Raw	SEM	Backfit	Recovery %
Standard1	A1	0	0.0579	0.0007	< Curve	-
	B1		0.0558		< Curve	-
	C1		0.0579		< Curve	-
Standard2	A2	7.55	0.112	0.00142	6.872	91.03
	B2		0.114		7.19	95.23
	C2		0.109		6.515	86.29
Standard3	A3	35	0.302	0.00215	38.32	109.5
	B3		0.309		39.66	113.3
	C3		0.303		38.48	109.9
Standard4	A4	75.5	0.449	0.00982	71.7	94.96
	B4		0.43		66.73	88.39
	C4		0.464		75.59	100.1
Standard5	A5	755	1.26	0.053	1372	181.7
	B5		1.14		692	91.65
	C5		1.08		555.5	73.58
Standard6	A6	7550	1.35	0.0435	3133	41.5
	B6		1.35		3071	40.68
	C6		1.48		> Curve	-



a	0.0468
b	1.01
c	902.8
d	1.57
MSE	0.003538
R^2	0.9862
SS	0.1698
SYX	0.06212

Calibrator	Wells	Conc.	Raw	SEM	Backfit	Recovery %
Standard1	A1	0	0.0474	0.000366	0.3871	-
	B1		0.0474		0.3871	-
	C1		0.0466		< Curve	-
	D1		0.0463		< Curve	-
	E1		0.0461		< Curve	-
	F1		0.0458		< Curve	-
	G1		0.0456		< Curve	-
	H1		0.0487		1.208	-
Standard2	A2	7.55	0.0543	0.000828	4.712	62.41
	B2		0.059		7.65	101.3
	C2		0.0558		5.65	74.83
	D2		0.0603		8.464	112.1
	E2		0.0553		5.337	70.69
	F2		0.0541		4.587	60.76
	G2		0.0565		6.087	80.62
	H2		0.0589		7.588	100.5
Standard3	A3	35	0.107	0.0047	38.55	110.1
	B3		0.105		36.71	104.9
	C3		0.101		34.23	97.8
	D3		0.101		34.1	97.43
	E3		0.139		59.65	170.4
	F3		0.1		33.9	96.87

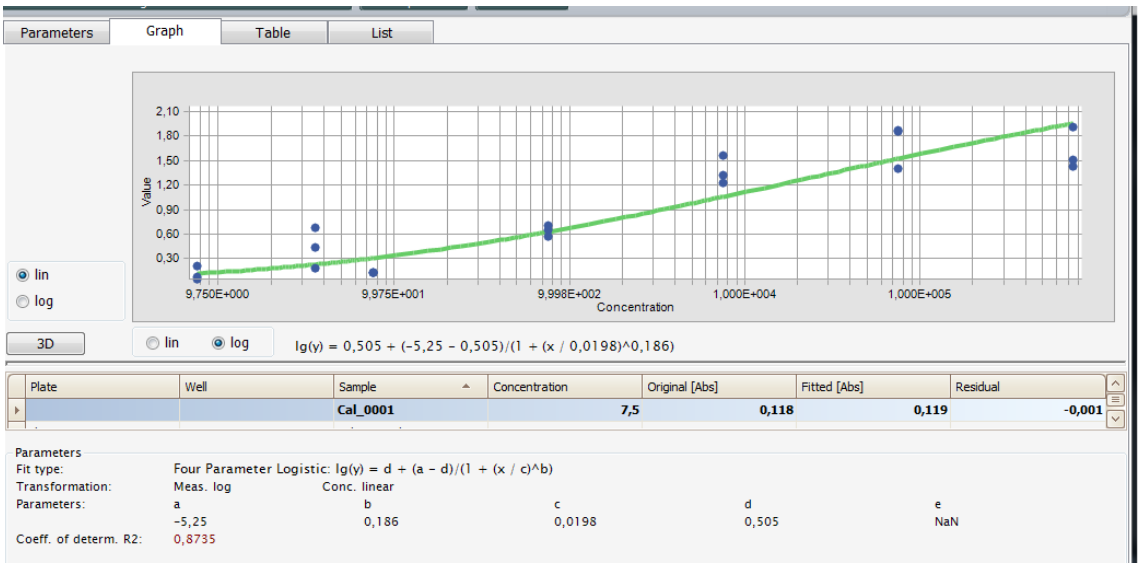
	G3		0.0996		33.51	95.75
	H3		0.101		34.36	98.17
Standard4	A4	75.5	0.203	0.00698	105.4	139.6
	B4		0.173		83.25	110.3
	C4		0.152		68.95	91.32
	D4		0.152		68.6	90.86
	E4		0.152		68.39	90.59
	F4		0.148		66.24	87.74
	G4		0.144		63.28	83.81
	H4		0.148		65.97	87.37
Standard5	A5	755	0.711	0.013	699.9	92.7
	B5		0.732		739.3	97.92
	C5		0.72		716	94.83
	D5		0.724		724.1	95.91
	E5		0.822		935.1	123.9
	F5		0.714		704.5	93.32
	G5		0.737		749.1	99.22
	H5		0.761		798.7	105.8
Standard6	A6	7550	1.39	0.0528	6706	88.83
	B6		1.67		> Curve	-
	C6		1.31		4355	57.68
	D6		1.31		4389	58.14
	E6		1.32		4457	59.04
	F6		1.63		> Curve	-
	G6		1.34		4953	65.61
	H6		1.31		4395	58.22



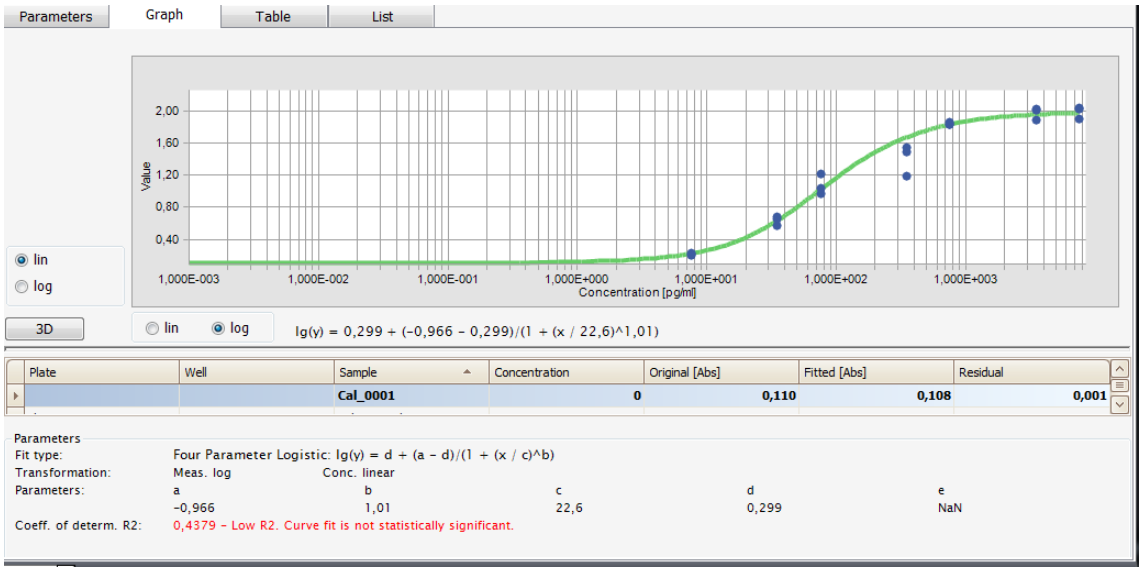
a	0.06163
b	1.036
c	435.5
d	1.279
MSE	0.0009924
R^2	0.9949
SS	0.01786
SYX	0.03572

Calibrator	Wells	Conc.	Raw	SEM	Backfit	Recovery %
Standard1	A1	0	0.0538	0.00491	< Curve	-
	B1		0.0555		< Curve	-
	C1		0.0693		3.288	-
Standard2	A2	7.55	0.0785	0.00333	7.089	93.89
	B2		0.088		10.99	145.6
	C2		0.0776		6.718	88.98
Standard3	A3	35	0.144	0.00145	34.68	99.09
	B3		0.149		36.82	105.2
	C3		0.148		36.16	103.3
Standard4	A4	75.5	0.246	0.00773	82.29	109
	B4		0.224		71.3	94.43
	C4		0.221		70.22	93.01
Standard5	A5	755	0.815	0.0379	696.5	92.25
	B5		0.789		638.3	84.54
	C5		0.914		987.4	130.8
Standard6	A6	7550	1.15	0.0379	3413	45.21
	B6		1.22		8455	112
	C6		1.28		> Curve	-

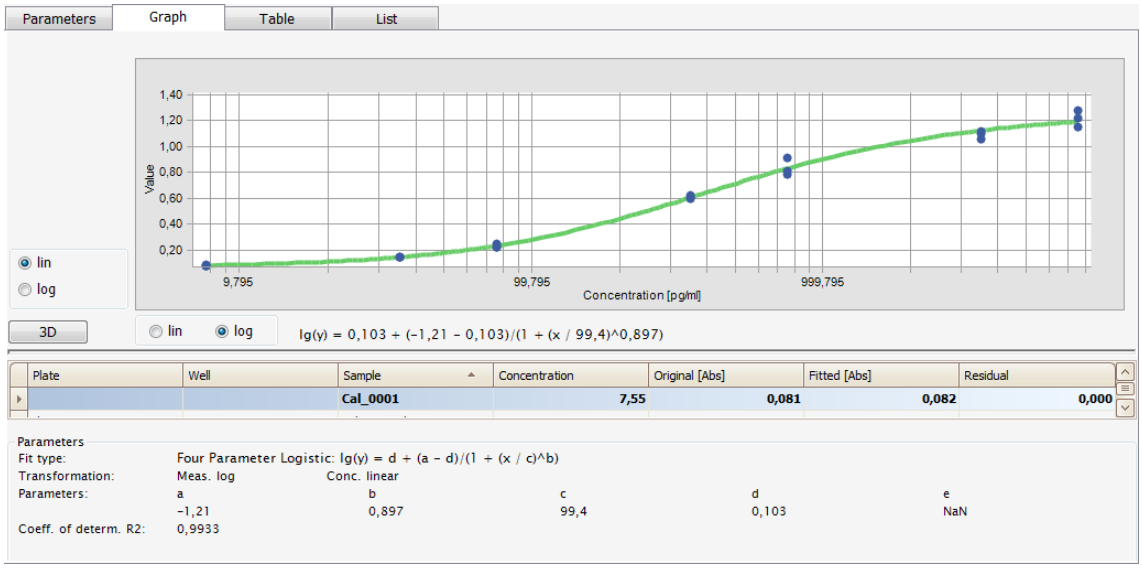
Recovery results and graphs



MILK, diluted with milk matrix					
spiked ricin pg/ml				average pg/ml	calculated from equation
0	NaN	NaN	NaN		1.262
7.55	NaN	NaN	NaN		1.548
75.5	7.546679	10591.97	NaN	7.547	6.420
755	114.3751	89.32504	141.02294	114.908	115.867
7550	18258.46	5710.232	6841.8294	6276.031	6445.660
75500	46144.66	213585.6	507634.03	360609.806	339663.023
755000	NaN	44065.39	48180.579	46122.982	47687.504
7550000	29090.77	36316.94	53031.918	39479.873	39568.510
WINE, diluted with wine matrix					
spiked ricin pg/ml				average pg/ml	calculated from equation
0	NaN	NaN	NaN		1.770
7.55	NaN	NaN	NaN		1.328
75.5	NaN	NaN	32.5421	32.5421	2.291
755	NaN	NaN	NaN		1.642
7550	NaN	NaN	NaN		2.145
75500	13.5720	53.7159	73.4959	63.6059	64.322
755000	25.8581	22.8660	10.5693	24.3620	24.774
7550000	2883.575	3988.179	4919.188	3930.314	3959.722
FLOUR, extraction with 2 % acetic acid					
spiked ricin pg/ml				average pg/ml	calculated from equation
0	NaN	NaN	NaN		1.737
7.55	NaN	NaN	NaN		1.673
75.5	NaN	NaN	NaN		5.261
755	NaN	NaN	NaN		2.208
7550	NaN	NaN	NaN		2.431
75500	13.37217	NaN	NaN	13.3722	6.289
755000	18.34444	26.36104	24.811859	25.5865	26.048
7550000	610.1975	509.1494	519.50476	546.284	558.702



	MILK, diluted with 0.03 % BSA 0.1% TritonX in PBS						
	spiked ricin pg/ml				average pg/ml	ricin c (pg/ml)	calculated from equation
	0	3.5384	2.6330	NaN	3.0857	30.8572	31.2
01:10	75.5	NaN	NaN	NaN			19.3
01:10	755	10.4548	11.6309	16.4019	12.829	128.2917	111.0
01:10	7550	74.9653	70.1782	319.0056	154.716	1547.1638	722.9
01:10	75500	1040.7190	394.6471	424.3185	619.895	6198.9489	4060.9
01:10	755000	NaN	NaN	4666.5913	4666.591	46665.9133	9555.3
01:10	7550000	NaN	NaN	NaN			7340.3
1/100	755000	971.9256	1300.6739	675.4849	982.695	98269.481	91501.1
1/100	7550000	NaN	NaN	7129.5733	7129.5733	712957.326	1122235.7
	WINE, diluted with 0.03 % BSA 0.1% TritonX in PBS						
	spiked ricin pg/ml				average pg/ml	ricin c (pg/ml)	calculated from equation
	0	NaN	NaN	NaN			33.4
01:10	75.5	1.0230	3.2322	0.6220	1.6258	16.2576	8.4
01:10	755	9.0401	17.0907	11.4236	12.518	125.1813	102.9
01:10	7550	77.7391	74.0635	97.5079	83.103	831.0350	756.1
01:10	75500	2458.6296	770.0737	520.4942	1249.733	12497.3251	6191.3
01:10	755000	3424.7704	NaN	1190.7872	2307.779	23077.7878	26862.3
01:10	7550000	NaN	NaN	NaN			17608.0
1/100	755000	2401.0550	673.4283	1837.8689	1637.451	163745.071	209962.3
1/100	7550000	NaN	NaN	NaN			248088.9
	WINE pH 8						
	spiked ricin pg/ml				average pg/ml	calculated from equation	
	0	NaN	3.8104	NaN	3.8104	2.1	
	75.5	NaN	NaN	0.1775	0.1775	0.03	
	755	1.7124	28.6806	13.7224	14.705	14.5	
	7550	28.8750	0.6007	1.9297	10.468	1.3	
	755000	217.5300	182.9462	179.4888	193.322	180.1	
	7550000	1403.3124	5785.5560	3699.4693	3629.446	4693.9	



FLOUR, extraction with PBS-tween					
spiked ricin pg/ml				average pg/ml	calculated from equation
0	17.9214	NaN	NaN	17.9214	0.4
7.55	NaN	NaN	NaN		1.9
75.5	NaN	9.0290	NaN	9.0290	1.4
755	NaN	NaN	7.9388	7.939	1.0
7550	32.7525	31.7010	27.9473	30.800	32.6
75500	331.9177	240.9892	244.1056	272.337	243.5
755000	NaN	1653.5475	1959.9018	1806.725	1802.0
7550000	NaN	5039.8073	NaN	5039.807	7147.3